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# BIOMARKERS IN *SOLEA SENEGALENSIS* KAUP, 1858 EXPOSED TO CONTAMINATED ESTUARINE SEDIMENTS: A MULTI-LEVEL APPROACH

Thesis submitted to the *Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia* for the degree of Doctor of Philosophy in Environmental Sciences.

This thesis was supported by a *Fundação para a Ciência e Tecnologia* fellowship, reference number SFRH/BD/28465/2006.

Lisboa

2011

## AUTHOR'S DECLARATION

The author states the he afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation, according to the *nº 2 do art 8º do Decreto-Lei 388/70*.

*Pedro Manuel Broa Costa*

*O sweet spontaneous*
*earth how often have*
*the*
*doting*

*fingers of*
*prutient philosophers pinched*
*and*
*pocked*
*,has the naughty thumb*
*of science prodded*
*thy*

*beauty* . […]

E.E. Cummings

To my father, who survived a PhD before me.

**Acknowledgements**

For their priceless support, a very special acknowledgement goes to my PhD supervisors, Professors Maria Helena Costa and T. Àngel DelValls. I have been given creative freedom however without ever being alone.

A great thanks goes to Sandra Caeiro, for her friendship and support throughout the WEP Sado project, and this thesis; for all her time, patience and friendship.

To my colleague, friend and, often, mentor, Mário Diniz, for all the help, all the work we've done together and all the great scientific debate.

To everyone else who worked in the WEP Sado project: a special thanks goes to Jorge "Cockle" Arteaga, Sónia Silva, Teresa Neuparth, Maria José Salamanca (and Mario!), Miguel Caetano, Marta Martins, Ana Ferreira and Carlos Vale.

A special word goes also to those from IPIMAR who gave their important contributions: Joana Raimundo, Rute Cesário, Vasco Branco and also Pedro Pousão (CRIP-SUL) and his group.

To the "chicas" from Cádiz: Rocío, Maria José, Laura and Julia.

To professor Lopez Barea, from the University of Córdoba, and his group, with my special thanks and friendship to Eduardo Chicano, José Alhama and all the others: Carlos, Ricardo, Inma and the lot.

To the Department's technical staff for all their vital assistance: Adélia, Luísa and others.

I should not forget to send a word of friendship to all the other "kids" that have been passing through our work group. On the very least, for their friendship: Tiago, Mafalda, Sofia, Joana, João.

I thank also Lia Ascensão, Luísa Mota and Francisco Carrapiço from FCUL for the help and debate on (among other things) histology and cytology.

To Célia Miguel and her group from ITQB for the most important assistance with the transcriptomic analyses.

To everyone from the DCV/UNL (former SABT/UNL) who gave me a very special help (including entrusting me some of the equipment...): Álvaro Fonseca, João Inácio, Marco Ferreira and others.

I must also thank Filomena Gomes (IMAR) for all the help.

To the RNES and APSS institutions (Setúbal) for all their support for the field work in the Sado Estuary.

At last (but certainly not the least), to all my family, especially to my wife, who endured everything by my side, including my ill-tempered disposition in the most difficult hours.

## Sumário

Os sedimentos estuarinos podem constituir um reservatório de contaminantes com diversas fontes, entre os quais se podem encontrar substâncias poluentes de origem antropogénica que mediante certas condições podem ser redisponibilizados para os organismos. No entanto, a avaliação da toxicidade dos xenobióticos associados aos sedimentos possui vários constrangimentos, relacionados maioritariamente com a complexidade geoquímica da matriz sedimentar e com a potencial existência de múltiplas classes de contaminantes. De forma a contribuir para uma metodologia de evidência ponderada para a determinação do risco ecológico de sedimentos estuarinos, foi empreendida uma bateria de bioensaios de 28 dias, realizados no campo e no laboratório, utilizando *Solea senegalensis* juvenis. Este conjunto de ensaios pretendeu contribuir com duas linhas-de-evidência para um processo de análise de risco ecológico no estuário do Sado: toxicidade e química sedimentar. Foram seleccionadas três estações (uma de referência e duas contaminadas), cujos sedimentos foram analisados para determinação de parâmetros físicos e níveis de contaminação (metálica e orgânica). Tendo sido examinado o fígado dos animais expostos como principal órgão-alvo, foram analisados vários biomarcadores, representando vários níveis de organização biológica: molecular, celular, histológico e do próprio órgão. Para o efeito, foram empregues várias metodologias, desde os clássicos métodos imunoquímicos e electroquímicos para determinação da indução de proteínas do citocromo P450 e metalotioninas, respectivamente; até às abordagens que reflectem o actual estado-da-arte na busca e validação de potenciais biomarcadores, nomeadamente as metodologias "ómicas" - toxicogenómica e proteómica; para além de métodos histopatológicos semiquantitativos e de determinação de genotoxicidade, entre outros. A integração das respostas biológicas com os parâmetros sedimentares revelou que, embora existam diferenças entre os ensaios de campo e de laboratório, os biomarcadores que reflectem lesões permitem distinguir de forma mais consistente os sedimentos contaminados dos de referência. Por outro lado, os marcadores moleculares permitiram estabelecer padrões de alterações do metabolismo e inferir como estas alterações podem afectar os mecanismos de resposta à exposição, da apoptose à defesa anti-oxidativa, transcrição génica, entre outros. A metodologia foi complementada com a realização de ensaios de laboratório com xenobióticos-modelo isolados ou combinados (cádmio + benzo[a]pireno), com o intuito de testar técnicas e estudar os complicados mecanismos de interacção de contaminantes. Os resultados demonstram que sedimentos moderadamente contaminados podem causar efeitos adversos nos organismos e despoletar respostas que reflectem os complexos mecanismos biológicos subjacentes à exposição a misturas de xenobióticos. A análise de múltiplos biomarcadores revela-se uma metodologia consistente quer para estudos de monitorização ambiental quer para fins mais mecanísticos, especialmente quando a existência de múltiplos contaminantes propicia o desaparecimento da especificidade das respostas.

## Abstract

Estuarine sediments can be a reservoir of contaminants from several sources, among which can be found pollutants of anthropogenic origin that, under certain circumstances, may be returned available to the biota. Nevertheless, assessment of the toxicological potential of sediment-bound xenobiotics has many constraints, especially related to the complex geochemical nature of the sediment matrix and to the potential existence of multiple classes of contaminants. In order to contribute to a weight-of-evidence approach to assess the ecological risk of estuarine sediments, an array of 28-day bioassays was enforced, *in situ* and in the laboratory, using juvenile *Solea senegalensis* as test subjects. The battery of assays aimed at contributing with two lines-of-evidence for an ecological risk assessment approach to the Sado Estuary, namely sediment chemistry and toxicity. For the purpose, three sites were selected (a reference plus two contaminated), from which sediments were collected and analysed for physical parameters and contaminant concentrations (both metallic and organic). Having the liver of tested fish been surveyed as the main target organ, several biomarkers were analysed, corresponding to different levels of biological organization: molecular, cellular, histological and organ itself. Several methods were employed, from the classic immune- and electrochemical methods to determine CYP1A and metallothionein induction, respectively; to more state-of-the-art approaches that are now being employed to find and validate new sets of biomarkers, namely “omics” - toxicogenomics and proteomics, besides semi-quantitative histopathological methods, genotoxicity assessment and others. The integration of biological responses with sediment parameters revealed that, although differences between *in situ* and laboratory assays exist, the biomarkers that in essence reflect some measure of lesion allow a much more consistent distinction between contaminated and uncontaminated sediments. In addition, molecular biomarkers permitted inferring patterns of metabolic change and assess how these changes contribute to the impairment of the response machinery to chemical insult, from apoptosis to anti-oxidative defence, among others. The multi-level approach was complemented by a series of laboratory assays with model xenobiotics, isolated or combined (cadmium + benzo[a]pyrene), aiming at technique testing and set-up and, most importantly, at surveying the complex mechanisms underlying contaminant interactions. The results demonstrate that even moderately contaminated sediments can cause adverse effects to organisms and trigger responses that reflect the intricate machinery beneath exposure to complex mixtures of xenobiotics, either for monitoring or for more mechanistic studies, especially when the existence of multiple contaminants tends to dilute biomarker specificity.

## Abbreviations

2DE - Two-dimensional electrophoresis

AB&NFR - Alcian blue and nuclear fast red histological stain

AI - Apoptotic indice

AO - Acridine orange fluorochrome

CASP3 - Caspase 3

CAT - Catalase

CBB - Coomassie brilliant blue (stain for peptides, G/R 250)

CYP1A - Cytochrome P450 1A

DNA-SB - DNA strand breakage

DPP-SMDE - Differential pulse polarography with a static mercury drop electrode

dw - Sample dry mass (sample dry “weight”)

Eh - Redox potential

ENA - Erythrocyte nuclear abnormalities

ERA - Ecological risk assessment

EST - Expressed sequence tag

FF - Sediment fine particle fraction (particle size < 63 μm)

GC-ECD - Gas chromatography with electron capture detector

GC-MS - Gas chromatography mass spectrometry

GLM - Generalized linear models

GPx - Glutathione peroxidase

H&E - Haematoxylin and eosin histological stain

HSP - Heat-shock protein

ICP-MS - Inductively coupled plasma mass spectrometry

IE - Immature erythrocyte

IEF - Isoelectric focusing

IT-MS/MS - Ion trap tandem mass spectrometry

LOE - Line-of-evidence

$L_s$ - Fish standard length

MALDI-TOF MS - Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MN - Micronucleus

MT - Metallothionein

MW - Molecular mass (molecular “weight”)

PAH - Polycyclic aromatic hydrocarbon

PCA - Principal component analysis

PCB - Polychlorinated biphenyl

PCD - Programmed cell death

*PEL* - Probable effects level sediment quality guideline

*PEL-Q* - *PEL* guideline quotient

PKD - Proliferative kidney disease

*pp*'DDD - 1,1-dichloro-2,2-bis[*p*-chlorophenyl]ethane

*pp*'DDE - l,l-dichloro-2,2-bis[*p*-chlorophenyl]ethylene

*pp*'DDT - l,l,l-trichloro-2,2-bis[*p*-chlorophenyl]ethane

qRT-RT-PCR - Quantitative real-time reverse transcription polymerase chain reaction

ROS - Reactive oxygen species

SCGE - Single cell gel electrophoresis ("comet") assay

SDS-PAGE - Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

*SQG* - Sediment quality guideline

*SQG-Q* - Sediment quality guideline quotient

SQT - Sediment quality triad

TBARS - Thiobarbituric acid reactive substances

*TEL* - Threshold effects level sediment quality guideline

TOM - Sediment total organic matter

TSB - total DNA strand breakage

VC - Variation coefficient

ww - Sample wet mass (sample wet "weight")

$ww_t$ - Fish total wet mass (fish total wet "weight")

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# Chapter 1. General introduction

### 1.1. Ecological risk assessment in estuaries: sediments as reservoirs of pollutants

The determination of sediment ecological risk in confined coastal areas such as estuaries has long been a challenge for ecotoxicologists. Since the beginning of human settlement on the coastlines, estuaries have been considered premium sites due to their importance for maritime and river transportation, fisheries, agriculture, access to freshwater and many other geographical advantages. Centuries later, especially from the Industrial Revolution onwards, the existence of large human settlements combined with being the gateway for that vital merchant route - the Sea - turned estuaries into ideal sites to implant all sorts of industrial activities, with environmental consequences that are, to our days, one of the most serious problems that humans have to deal with for generations to come. With respect to pollution, estuaries tend, therefore, to be a repository of chemical contamination from diverse, but equally relevant, sources: industrial, urban, shipping and agricultural, or even by naturally-occurring substances that are washed-off with the river flows, having been released from eroded inland geological formations. These contaminants, on their turn, tend to be trapped in the complex geochemical matrix that characterizes estuarine sediments, sorbed to fine particles, organic matter or simply dissolved in pore water, through intricate partitioning processes that depend on the substances' type as well as the media's characteristics. Estuarine sediments are, therefore, potential reservoirs for contaminants. The trapped substances may enter the biota directly, for instance by action of deposit-feeders or, under certain circumstances (such as sediment disturbance), may be released back to the water column, turning them more readily bioavailable to organisms (see for instance Luoma, 1989; Eggleton and Thomas, 2004). In either case, besides direct toxicological and ecological effects, contaminants may be biomagnified throughout the food chain, on top of which are found the human populations that exploit estuarine resources. Special, but common, events in estuaries such as dredgings (for instance to enlarge or maintain navigation channels) are practical examples of the need to assess sediment risk, not only due to the potential hazard of the disposed materials but also due to the disturbance of deposited sediments with subsequent release of contaminants to the estuarine ecosystem (e.g. DelValls et al., 2004). Natural erosion of the upper layer of sediments in coastal water bodies (driven by tides, storms and heavy runoffs, for instance) is also a concern with respect to the release of contaminants back to the water columns (see for example Zimmerman et al., 2008).

Assessing the toxicological risk of estuarine sediments (or aquatic sediments in general) has, however, many constraints. These sediments have, as aforesaid, a complex geochemistry derived from multiple factors, of which the existence of multiple classes of toxicants, the complicated anoxia/hypoxia conditions, organic matter content and the naturally-occurring changes that affect labile environments such as estuaries are only examples. For all these reasons researchers have long attempted to implement methods that, following more complete, "holistic", approaches, allow a more substantiated approach to understand and predict the risk associated with sediment-bound contaminants in aquatic ecosystems. Such methods take part in a process commonly known as ERA:

Ecological Risk Assessment. One of the most consensual approaches to ERA has been the Sediment Quality Triad concept (SQT); first introduced by Long and Chapman (1985) in the process of risk assessment for the Puget Sound Basin (Pacific coast of the USA). The three components, or "Lines Of Evidence" (LOEs), of the SQT are: toxic effects to organisms; sediment contaminant concentration and changes to the ecosystem's macrofaunal structure (Fig. 1.1.1).

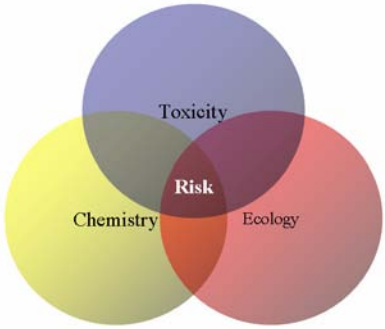


**Fig. 1.1.1.** Components or "Lines Of Evidence" (LOEs) of the Sediment Quality Triad.

Chapman and his co-workers (2002) have drawn a very clear distinction between "toxicity" and "risk": toxicity is a biological response that, when measured, might give an insight on the potential risk of a sediment to an ecosystem and to the human population that relies on this same ecosystem. In another words, the fact that toxicity testing showed that one or more substances may cause adverse effects to an organism does not necessarily mean that these same substances, in a given sediment, can actually cause negative effects to organisms. In the same paper it is also explained that contaminant concentrations in the sediments *per se* may not predict effects to organisms perhaps unless present in extreme, high or low, levels and, similarly, analysis of community structure (the ecological component of the Triad) may not, on its own, be sufficiently informative since communities tend to stabilize when affected by pollution, even if that means a complete change of its composition and structure, thus diluting the human understanding of "negative" and "positive" effects. In addition it has to be kept in mind that contamination does not necessarily mean pollution, i.e., pollution exists when it is proven that the contamination levels in the environment actually exert negative effects to the biota. Regardless of the discussion around the need to add more LOEs to enforce effective ERA programmes (see, e.g., Chapman and Hollert, 2006), there is a generalized consensus that sediment risk assessment is a multi--disciplinary and rather complex task to which should yet be devoted much research, especially if, in a near-future perspective, efficient regulatory policies are intended.

The present work will be based on two LOEs of the triad: toxicity and sediment chemistry, with a special focus on the measurements of toxicity, their biological significance, effective predictive value and their relationship with the observed sediment characteristics. The ecological structure of the study area, like in any other similar situations, would demand such an extensive work that it would

require a totally dedicated research programme on its own and is, therefore, out-of-scope at the present.

### 1.2. Toxicity testing: bioassay and biomarker approaches

Since sediment chemistry on its own can predict neither adverse effects to organisms nor mid- to long-term changes to the ecological structure of the environment, much attention has been given to the detection of such effects on organisms directly, either by surveying field-collected organisms or through experimental procedures based on bioassays. However, besides the simple survey for detrimental effects to organisms (and it potential extrapolation for community-level), researchers and managers have been trying to develop a set of biological effects and responses that might be used to indicate exposure to hazardous concentrations of substances. These changes are termed ecotoxicological biomarkers and rely on the very same principles that biomedical biomarkers rely on in order to indicate health changes caused by a particular illness. Thus, effective biomarkers are those that can be used to establish cause-effect relationships which, under the ecotoxicological point-of--view, means that biomarkers should be good indicators of pollution.

The biomarker approach is nowadays broadly recognized as an important tool for ERA, regardless whether more predictive or more mechanistic surveys are at stake. It is generally accepted that biomarkers consist of sub-lethal “early warnings” to the potential adverse effects to organisms caused, in this case, by environmental xenobiotics (see, for instance, van der Oost et al., 2003, for a review). The standard definition for “biomarker” in environmental research, as defined by van Gestel and van Brummelen (1996) stands for any sub-individual level alteration that reflects exposure to a contaminant. These “changes” are consensually regarded, therefore (or expectedly), as measures of toxicity. The same authors also state that the term should not be confused with “bioindicator” (an organism whose presence, absence or altered behaviour can indicate change in ecosystem quality) and “ecological indicator” (which is an ecosystem- and not individual-level effect - Fig. 1.2.1). Traditionally, biomarkers are divided in two major categories: biomarkers of exposure and biomarkers of effect, even though this classification is not always consensual, especially since it does reflect more the way how biomarkers are interpreted than their actual biological meaning. Biomarkers of exposure are normally considered to relate to the detection of induced substances or metabolites while biomarkers of effect should reflect changes to cells, tissues, organs and fluids. A third category, biomarkers of susceptibility, which concern to the organism’s ability to respond to a potential challenge, can also be added to the list (refer to Martín-Díaz et al., 2004, for a review). It should be noted that bioaccumulation is not regarded as a biomarker since the presence of contaminants (or their metabolites) in tissues does not necessarily mean adverse effects. However, it can, under certain circumstances, indicate the presence of such substances in the environment (van Gestel and van Brummelen, 1996; van der Oost et al., 2003). In general, still, bioaccumulation tends to be very

variable for being impacted by many factors: both external, such as those affecting bioavailability, and internal, like as the ability of many organisms to rapidly catabolize organic substances like polycyclic aromatic hydrocarbons and others (e.g. Varanasi and Gmur, 1981; Luoma and Rainbow, 2005; Ruus et al., 2005).

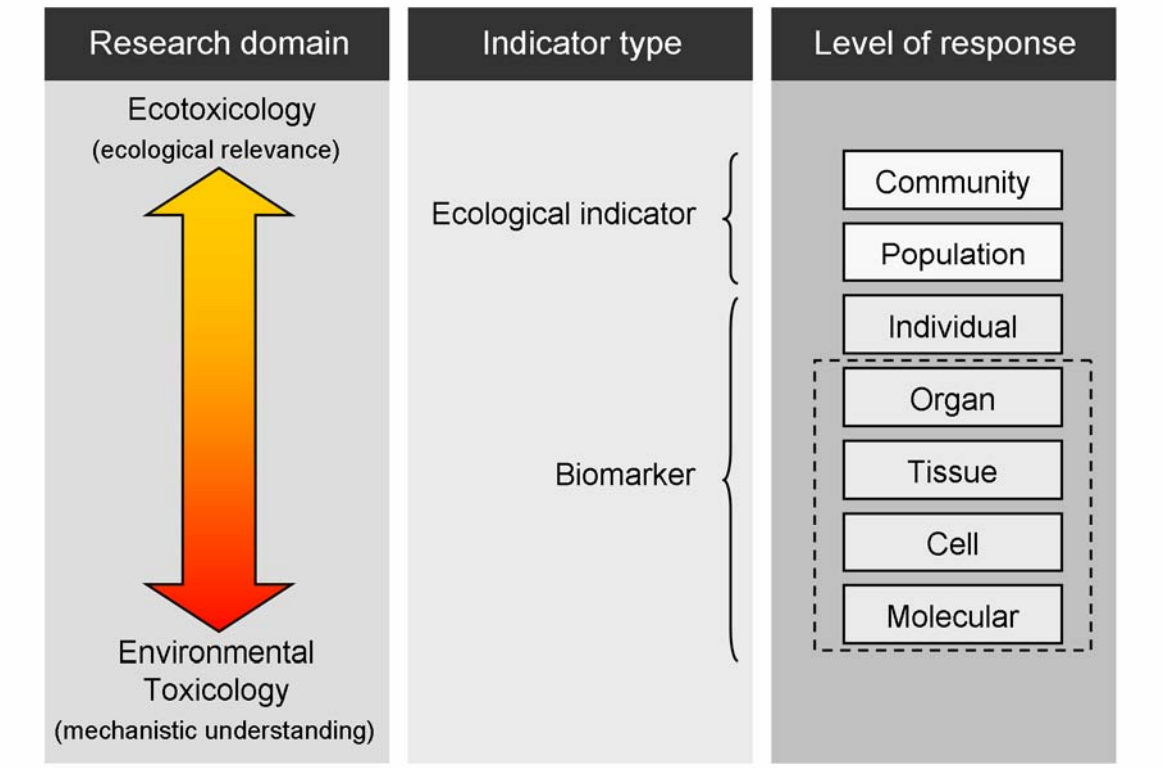


**Fig. 1.2.1.** Theoretical organization of biological indicators of contamination in environmental research, highlighting the levels of response (i.e., the levels of biological organization) within the scope of the current dissertation (dashed box). Diagram partially adapted from Hinton et al. (2005).

The combination of bioassays using aquatic organisms with biomarker response measurements has been the most broadly-enforced approach to toxicity testing for being informative, expedite and cost-effective (Lam and Gray, 2003). Bioassays, on their turn, are experimental procedures (opposed to surveying feral organisms) that potentially allow a better understanding of cause-effect relationships through the possibility of manipulating the adequate variables (Chapman, 2007). Bioassays can be performed in controlled (laboratorial) environments or *in situ* (field) and may be used to test single or mixed contaminants, waterborne or sediment-associated - obtained either from artificial (e.g. spiked sediments) or natural media (like water samples or sediment batches collected with a grab, core or other). It is generally recognized that laboratory bioassays eliminate environmental confounding ("noise") variables, in theory permitting to establish a clearer link between contamination and toxicity. Nevertheless, it is also acknowledged that laboratory studies may either underestimate or overestimate toxicity, rendering the results less ecologically relevant than *in situ* procedures (Martín-Díaz et al., 2004; Chapman, 2007). The choice of the approach relies, therefore, on the fine balance between

objectives, logistics and the need to safeguard realism without compromising the quality of the results. Very little research, however, has objectively focused on the direct comparison between the two types of assays.

With respect to testing multi-class mixtures of contaminants, furthermore if present in complex media such as in natural sediments, much research reports inconclusive results or even the inadequacy of surveying "classic" biomarker responses such as CYP1A and metallothionein (MT) induction (for instance Hylland et al., 1996; Mouneyrac et al., 2002; Costa et al., 2008; Leaver et al., 2010). In the past few years a considerable amount of literature sprung out to address the issues of bioavailability modulation and contaminant interaction effects using many different *in vivo* and *in vitro* biological models. Among these studies there can be found, for instance, researches focusing on the release of contaminants from sediments as a results of disturbance with subsequent increase in bioavailability (e.g. Brinkmann et al., 2010), or the effects of combined exposures to different xenobiotics and its consequence on biomarker responses as a consequence of additive, antagonist of synergistic effects resulting from co-exposure (e.g. Spink et al., 2002). The information resulting from these studies will be discussed in detail throughout the present dissertation.

The complexity of testing the toxicity of environmental samples (as stated, sediments and water - or even their combination, as in field assays) is leading ecotoxicologists to a more integrative, multi-biomarker approach. This strategy may aim at finding which biomarkers retain their specificity under realistic environmental conditions but, especially, it has been aimed at gathering patterns of response that reflect overall hazard without the limitations imposed by measuring a specific response that by failing to produce convincing results may compromise the whole study. Such strategies have become more and more common in studies with more practical goals in biomonitoring programmes (for instance Montes-Nieto et al., 2010; Wenger et al., 2010). However, regardless if it is intended a more predictive (for ERA) or more mechanistic (for fundamental toxicology) purpose, there is still a great need for further research.

Finally, the difficulties in establishing cause-effect relationships, by all the aforesaid reasons, when testing environmental samples, combined with the availability of novel, state-of-the-art, molecular tools, brought biomarker assessment to a whole new level in the past decade, especially with the introduction of "omic" high-throughput approaches (proteomics, transcriptomics and toxicogenomics) into the field of ecotoxicology. These techniques have many advantages, although they are not yet neither standardized nor cost-effective, among which there is the possibility of surveying multiple responses or changes without the need of much *a priori* knowledge about the biological mechanisms of response to toxicity. Furthermore, the integration of multiple responses should allow finding novel biomarkers, more adequate for a specific contaminant in a mixture or even specific to a given mixture of substances, besides providing new valuable insights on the complex, and yet largely unknown, pathways of response to chemical insult (see, e.g., Gatzidou et al., 2007; Monsinjon and Knigge 2007, for reviews).

### 1.3. Flatfish in Ecotoxicology: the case of *Solea senegalensis*

The choice of the appropriate target organism is one of the most important issues when developing a research on environmental monitoring and toxicology. Piscine species have long been regarded as selection test subjects to aquatic toxicology due to their abundance, diversity (representing about half of all known vertebrate species), ecological and economical relevance, availability (e.g. through facilitated brooding) and morphological similarity with other (vertebrate) models. Consequently, teleosts appeared as convenient subjects in a variety of ecotoxicological scenarios, from single-substance testing to the monitoring of environmental quality (see Bolis et al., 2001). In addition, due to their increased sensitivity to toxicants, severity of toxicological effects on development and the ecological consequences of impaired recruitment and settlement, many ecotoxicological studies have focused on early life stage fish, from larvae to juveniles (Rolland, 2000; Ábalos et al., 2008; Costa et al., 2008; Oliva et al., 2009 and many others).

Among teleosts, flatfish (Teleostei: Pleuronectiformes) have long been employed in active biomonitoring procedures, with proven success. Among these procedures, it should be highlighted the analysis of feral flatfish for malignancies as a measure of sediment contamination (e.g. Myers et al., 1998; Koehler, 2004; Lang et al., 2006). In general, there has been a growing interest in this group of teleosts that is transversal to many areas of biological research, from ecology to fisheries, aquaculture and ecotoxicology. The attention given to flatfish species reflects in essence its ecological importance, economical value and, in particular for ecotoxicologists, its benthic behaviour (in addition, there is often fish availability from mariculture facilities). Although yet much incipient, a number of novel, state-of-the-art scientific resources is beginning to arise for these animals and is able be applied in all the aforesaid areas of research, among which “omics” (especially transcriptomics and proteomics) occupy a key position (Cerdà et al., 2010).

The Senegalese sole (*Solea senegalensis* Kaup 1858; Teleostei, Pleuronectiformes; Soleidae) is a common flatfish in southwestern Europe (Fig. 1.3.1). This benthic fish inhabits sandy or muddy floors on coastal areas, where it preys on small invertebrates, often inhabiting estuaries (including the Sado Estuary where it is an ecologically important species and at least a valuable by-catch for local fisheries) that are of importance as breeding and nursing grounds (Cabral and Costa, 1999; Cabral, 2000; Sá et al., 2003). In addition, the species has a high aquaculture potential in the Iberian Peninsula (Dinis et al., 1999).

The Senegalese sole has been successfully employed in toxicity testing of contaminated areas and of waterborne or intraperitoneally-injected substances, in both field and laboratory assays, and a considerable number of publications focusing on “classical” biomarker responses have been published in the past few years (for instance Arellano et al., 1999; Riba et al., 2004; 2005; Salamanca et al., 2008; Oliva et al., 2009), excluding the ones deriving from the present work (refer to Annex 1). These studies may indicate that *S. senegalensis* has the potential in southeastern Europe that another flatfish species, *Platichthys flesus*, has received in the northwest for the environmental monitoring of marine

and estuarine environments. It is clear, however, that much research is still needed to better frame the biology of flatfish (and soleids in particular) within practical ecotoxicological contexts, especially regarding biomarker assessment and evaluation in intricate toxicological scenarios, biological responses and defences to toxicity and, most particularly, to reinforce the genomic and proteomic knowledge that is still lacking, an issue that renders still in an embryonic stage most of the molecular tools available for these animals (Cerdà et al., 2010). In fact, teleost genomes still lack the degree of sequencing and annotation that model vertebrates benefit from, perhaps with the exception of the zebrafish (*Danio rerio*). Nevertheless, "omic" approaches have recently been developed for *S. senegalensis*, although the great majority for aquaculture-related research (Forné et al., 2009; Osuna--Jiménez et al., 2009; Prieto-Álamo et al., 2009; Salas-Leiton et al., 2009), which again reinforces the high potential of the species as test subject.

**Fig. 1.3.1.** Juvenile *Solea senegalensis* sampled on the course of the present research (≈ 10 cm standard length).

### 1.4. Introduction to the study area

The Sado Estuary (W Portugal) is a large basin of great ecological, social and economical importance. The estuary is historically subjected to many sorts of anthropogenic usage and alteration. The area comprises a fairly large city (Setúbal, with an important commercial harbour) with a dense heavy-industry belt that includes chemical plants (such as fertilizer production), a paper mill, a large thermoelectrical unit, shipyards, ore deployment facilities and others. It is also important for fisheries (one of the key components of the local economy), tourism and aquaculture activities. The importance of the estuary for maritime transport leads to frequent dredging operations to expand wharfs and maintain navigation channels. Runoffs from the extensive agricultural grounds located upstream also contribute to the transportation of xenobiotics (chiefly pesticides and fertilizers) to the Sado estuarine basin. In addition, the river itself is a significant source of metals to the estuary by crossing an important pyrite mining region (Cortesão and Vale, 1995). Also, it must be noted that a large portion of the estuary is classified as a natural reserve (Fig. 1.4.1). The conflict between the importance of its socio-economical activities and the need to protect the environment urges the need of implementing effective biomonitoring of contaminated sediments of the estuary - a strategy that has been developed

in previous research constituting, in part, the basis of the current dissertation with respect to the general characterization of the area (refer to Caeiro et al., 2009).

In the study area, three sites were chosen according to published research (Caeiro et al., 2005; Neuparth et al., 2005; Costa et al., 2008) that resulted from previous research projects: a reference site, located off the Tróia Peninsula, the farthest from pollution sources, and two potentially contaminated sites, located near Setúbal's harbour and off the city's heavy-industry belt. Although the estuary has been regarded as moderately contaminated (see Caeiro et al., 2009, and references therein), the selected contaminated area comprises stations where some of the highest levels of contaminants (organic and metallic) were found, in part due to the proximity of Setúbal's heavy-industry belt, in part caused by the hydrodynamic characteristics of the area, which permits, especially near the city's harbour, deposition of suspended fine particles and/or organic matter to which non-dissolved xenobiotics are mostly adsorbed to. However, as an example, a recent study aimed at surveying the levels of mercury in the estuary (in water, suspended matter and sediments) reported moderate contamination by this metal, including off the industrial belt here regarded as target site (Lillebø et al., 2010). Similar results were found in the past regarding the distribution of the organochlorine pesticide DDT in the estuary's sediments (Gil and Vale, 1999).

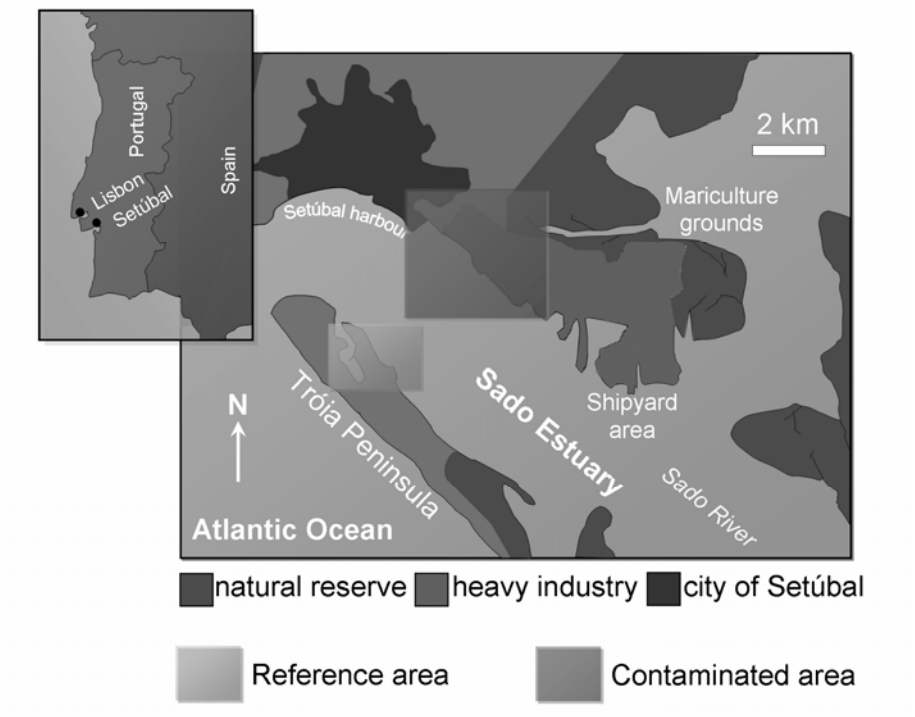


**Fig. 1.4.1.** Map of the study area of the Sado Estuary highlighting the reference and contaminated areas from which the surveyed sites were selected.

In spite of the acknowledged overall moderate levels of contamination, adverse effects to organisms have been found to result from exposure to sediments from the estuary, especially those collected at the contaminated area under survey in the present work. It is the case of a series of bioassays with the amphipod *Gammarus locusta*, where negative effects were found regarding DNA integrity and lipid peroxidation, to which is added a MT response linked to metal exposure (Neuparth

et al., 2005). Besides this biomarker approach, endpoints that can be regarded as being at populational level also revealed negative effects on *G. locusta*, namely survival, growth and reproduction (Costa et al., 2005). These studies not only disclosed that global moderate contamination can indeed elicit adverse effects to organisms (and, moreover, populations) but also showed that sediment testing in general, and for ERA in the particular case of the Sado Estuary, needs to be further developed, in part due to some inconsistent results regarding both biomarker and reproduction responses as a consequence of exposure to sediments (op. cit.).

### 1.5. Thesis layout and main objectives

The schematic organization of the thesis is presented in Fig. 1.5.1. The main objectives of this thesis are summarized as follows:

**1)** It is aimed to determine the effects and responses of sediment-bound estuarine contamination to a benthic fish (the Senegalese sole) at various levels, from DNA and gene expression (transcription plus protein induction) to cell, tissue and organ degrees of biological organization and potential extrapolation to organism-level effects.

**2)** To integrate sediment chemistry (contaminant levels and other parameters) with the measured toxicological traits in order to infer the effective toxicity of the bioavailable fraction of the xenobiotics as a potential LOE for the study area.

**3)** To assess the value and significance of each toxicological trait analyzed as a potential biomarker to individual or combined sediment-bound contaminants.

**4)** To use the surveyed toxicological traits, and explore new frontline methodologies, to contribute to the understanding of the mechanistics of toxicity in juvenile Senegalese soles.

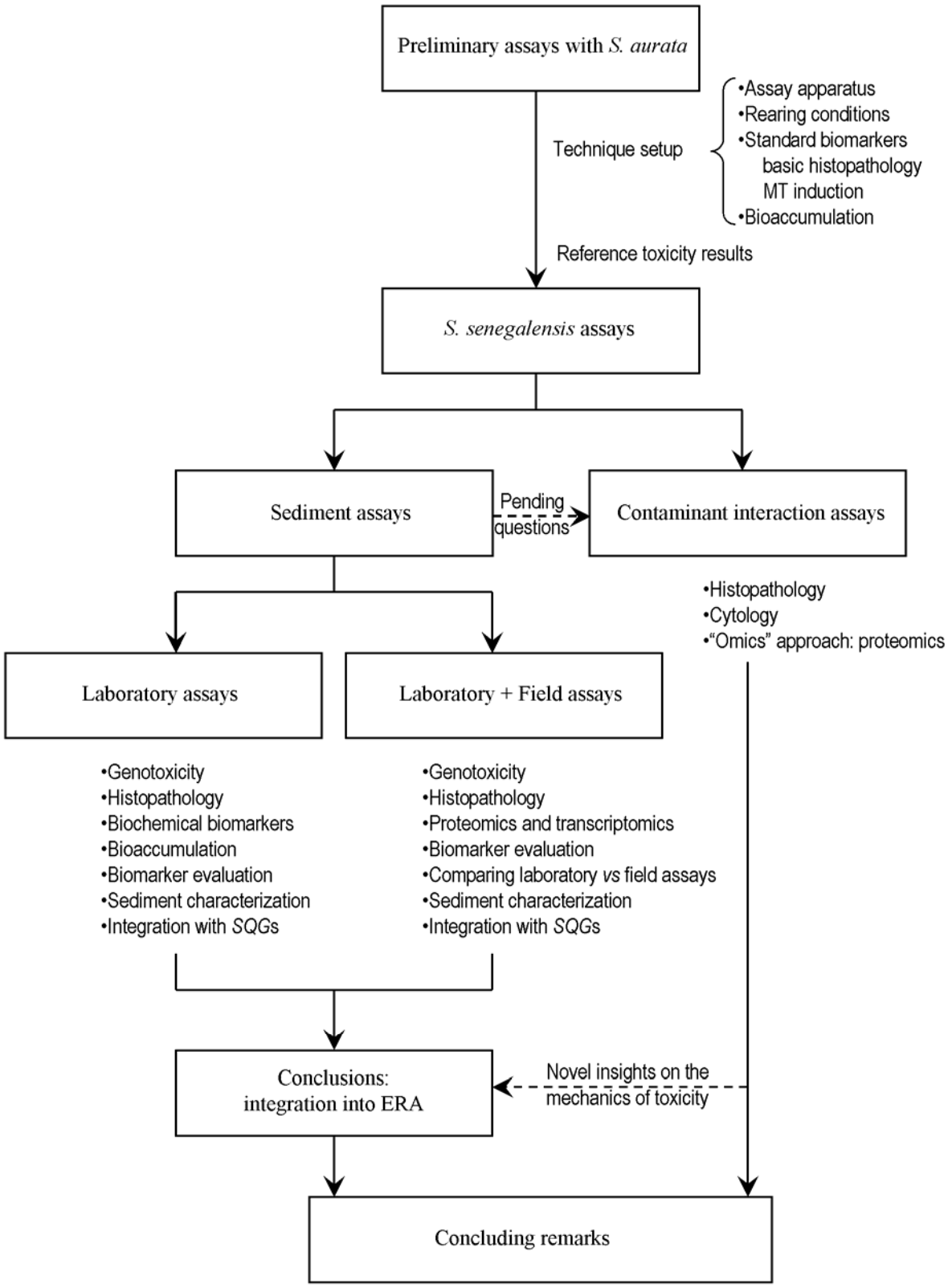


**Fig. 1.5.1.** General organization of the research and thesis highlighting the main analytical techniques and goals. ERA, ecological risk assessment; MT, metallothionein; *SQG*, sediment quality guideline.

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# Chapter 2. Baseline toxicity endpoints in fish and technique set-up

## 2.1. Genotoxicity assessment in fish peripheral blood: a method for a more efficient analysis of micronuclei[†]


### Abstract

The authors describe a method for analysis of micronuclei using a nucleic acid-specific fluorochrome, acridine orange, and ultraviolet microscopy in order to establish a simple and reliable technique for routine genotoxicity assessment in fish peripheral erythrocytes.



### Key-Words

Acridine orange; Fish peripheral blood; Genotoxicity; Micronuclei; Ultraviolet microscopy.


### 1. Introduction

Analysis of micronuclei and other nuclear deformities is a common protocol to evaluate genotoxic effects on animal cells, such as hepatocytes and gill, kidney, bone marrow or blood cells. In fish, analysis of micronuclei in blood cells is often used as a convenient biomarker of exposure to several types of environmental contaminants, under controlled or natural conditions, since fish erythrocytes are nucleated (Rodriguez-Cea et al., 2003; Çavaş et al., 2005; Baršienė et al., 2006; Bolognesi et al., 2006). Nevertheless, the lack of uniformity of the criteria to assess nuclear alterations in fish blood cells and the difficulty in interpreting objectively the observed occurrences are decisive factors that often relegate this analysis to a secondary role. Classic staining procedures, such as May--Grunwald's Giemsa, routinely used in bright-field microscopy, are often feeble and time-consuming regarding analysis of micronuclei. Artefacts are frequently difficult to discriminate from micronuclei or other abnormalities and, for such reason, multiple readings by multiple observers or blind reviews of coded slides by the same observer are frequently employed. Some authors quantify total 'nuclear abnormalities' rather than discriminate micronuclei and other alterations of the nucleus (see for instance Çavaş and Ergene-Gözükara, 2005).

Acridine orange (AO) is a nucleic acid-specific ultraviolet fluorochrome that stains DNA bright yellowish-green and RNA orange/reddish on a typically green cytoplasmic background. This

[†] Costa & Costa (2007). *J. Fish. Biol.* **71**(SA), 148-151 (doi:10.1111/j.1095-8649.2007.01548.x).

specificity eliminates common non-nucleic acid artefacts (such as protein granules) that are common confounding factors in bright-field microscopy, thus allowing faster and more error-free scorings. Nevertheless, analysis of micronuclei in fish using ultraviolet microscopy is not routinely employed, an exception being the work by Bolognesi et al. (2006). Furthermore, analysis of micronuclei in fish blood still lacks references on protocol, blood cell types and nuclear abnormalities. This paper aims to establish fluorescence microscopy based on AO staining as a viable routine procedure for analysis of micronuclei in fish erythrocytes and to present a visual reference on most common nuclear abnormalities found using this method.

## 2. Methods and Materials

Juvenile (≈ 80 - 100 ± 14.6 mm standard length, 20 - 30 g total wet weight) laboratory-reared gilthead seabream (*Sparus aurata* L., 1758) were injected intraperitoneally with 500 μL cadmium (Cd) standard solution (Merck) diluted in 50 mM phosphate buffer, pH 7.4 (0.4 $mg.mL^{-1}$ Cd) to induce formation of micronuclei, since Cd is a well-known genotoxic agent in fish (Ayllon and García--Vázquez, 2000; Çavaş et al., 2005). Control individuals were injected only with phosphate buffer. Blood was collected with a syringe from the caudal peduncle 24 h after injection and smeared on glass microscopy slides. Slides were allowed to air-dry overnight and were fixed in absolute methanol for 15 min and again air-dried overnight. Slides may be safely stored after this step: prolongation of the pre-fixation period promotes DNA and RNA degradation and consequently causes loss of staining effectiveness. Slides were directly dipped in an opaque glass Hellendahl jar, containing cold staining solution for 30 min. Staining solution was prepared with 0.1 $mg.mL^{-1}$ AO base (from Sigma; maximum absorbance 488 nm) in distilled water to which glacial acetic acid (1% v/v) was added. Staining solution was stored in the dark at 4 °C and proved to be effective for more than 5 weeks. Immediately after staining, slides were very briefly rinsed with washing solution (0.5% v/v glacial acetic acid in absolute ethanol) and dipped in 100% ethanol for 1 min, followed by 1 min in xylene. Slides were then allowed to air-dry overnight (in the dark), mounted with DPX (BDH, Poole, England), a non-fluorescent mounting medium, and were stored in the dark. Although staining tends to fade upon exposure to ultraviolet light, staining is persistent for an indefinite period of time.

Slides were observed with a BX50 microscope adapted for epifluorescence using a U--ULH100W mercury high-pressure bulb and a U-MWB filter (Olympus, Tokyo, Japan). Erythrocytes were observed and counted from colour microphotographs (× 1,000 magnification). Cells from two control and two contaminated individuals were surveyed (two slides each), and approximately 1,000 intact cells per individual were counted. Counting of nuclear abnormalities follows classification proposed by Fenech et al. (2003) for analysis of human lymphocyte micronuclei. Fish blood cell classification follows Yokote (1982).

### 3. Results and Discussion

Most erythrocytes observed presented normal nuclei (Fig. 2.1.1a). Immature erythrocytes Fig. 2.1.1b) are characterized by a more spherical shape and a larger and rounder nucleus than mature erythrocytes and also by a narrower, yellowish cytoplasm. Thrombocytes (Fig. 2.1.1c) and defence cells, mostly neutrophils and lymphocytes, were also present (Fig. 2.1.1d, e). According to the findings, neutrophils typically present an orange-red colouration that may be due to higher quantities of RNA in the cytoplasm. True micronuclei (Fig. 2.1.1k) in erythrocytes (fully detached, with a diameter less than one-third of the main nuclei) were found to be rare even in exposed individuals, but other nuclear abnormalities were common and represented the large majority of nuclear alterations (Fig. 2.1.1f-j). Within typical abnormalities, it was possible to observe several types of 'lobed' nuclei that corresponded to budding or fragmenting nuclei. Budding nuclei may be the first stages in formation of micronuclei, whereas fragmentation may originate polynucleated cells. The authors also observed nuclei that simultaneously presented various types of abnormalities, such as nuclear buds combined with fragmentation (Fig. 2.1.1j). Erythrocytes from contaminated individuals frequently exhibited corrugated membranes (Fig. 2.1.1f-j) or even a spherical shape (Fig. 2.1.1k), which may be caused by Cd exposure, as described by Nikinmaa (1992). Spherical mature erythrocytes are discriminated from immature erythrocytes by different AO staining of the cytoplasm. The results are summarized in Table 2.1.1.

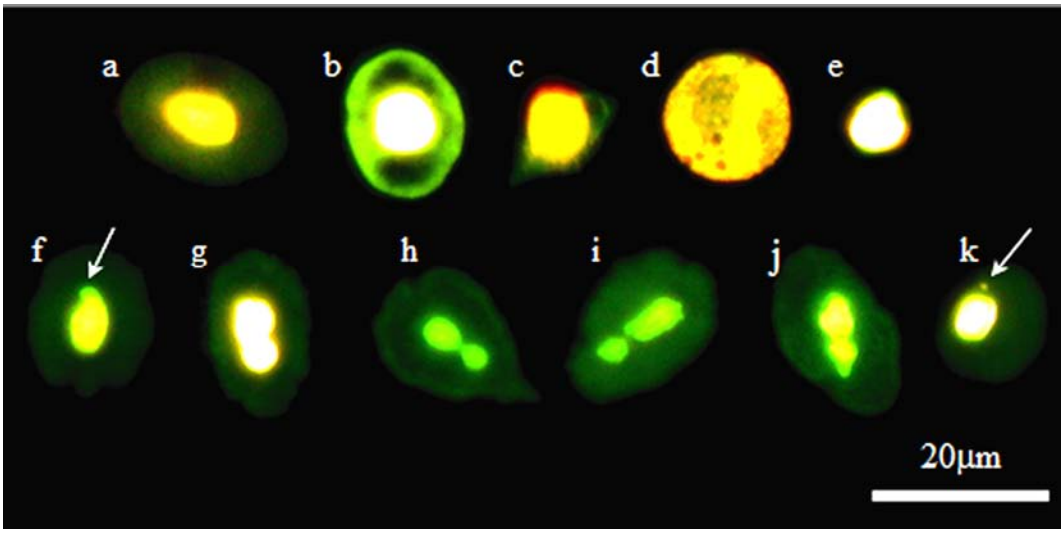


**Fig. 2.1.1.** *Sparus aurata* peripheral blood cells (AO, × 1,000). a-e) normal cells of control individuals: a) mature erythrocyte; b) immature erythrocyte; c) thrombocyte; d) neutrophil and e) lymphocyte. f-k) nuclear abnormalities in mature erythrocytes of Cd-injected individuals: f) nuclear bud (arrow); g) and h) fragmenting nuclei; i) fully fragmented nucleus (binucleated cell); j) multiple nuclear abnormalities: fragmentation and formation of nuclear buds and k) micronucleus (arrow).

**Table 2.1.1.** Summary of nuclear abnormalities percentages found in blood of Cd-injected and control individuals.

| | Injected Cd dose ($\mu g.g^{-1}$ fish $ww_t$) | Micronuclei (%) | Total nuclear abnormalities (%) | Cells counted |
|---|---|---|---|---|
| Cd | 6.8 | 1.4 | 17.4 | 1,000 |
| | 4.7 | 1.0 | 5.3 | 1,000 |
| Control | - | 0.1 | 5.0 | 1,008 |
| | - | 0.4 | 4.9 | 1,001 |

$ww_t$, fish total wet weight.

Due to the specificity of AO staining to DNA and RNA, discrimination of nuclear abnormalities proved to be less time-consuming and more accurate than traditional bright-field techniques, since non-nucleic acid artefacts are not stained and there is a better contrast to discriminate small structures, such as micronuclei and nuclear buds. The authors suggest that this approach is a simple, fast and more reliable genotoxicity assessment method for fish erythrocytes compared to traditional bright-field microscopy.

## Acknowledgements

The research was approved by the Portuguese Science and Technology Foundation and POCTI (Programa Operacional Ciência, Tecnologia, Inovação; Research Project POCTI/AMB/57281/04) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors would like to thank Isabel Spencer, Álvaro Fonseca, José Mário Sampaio and João Inácio (DCV - FCT/UNL) and Mário Diniz (REQUIMTE - FCT/UNL) for their support.

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## 2.2. Biochemical and histopathological endpoints of *in vivo* cadmium toxicity in *Sparus aurata*[†]


### Abstract

Cadmium (Cd) is a non-essential metal common in water bodies subjected to anthropogenic pollution. Its proven toxicity to aquatic and terrestrial organisms (including humans) has made this metal a subject of particular interest in toxicological studies, especially concerning common coastal fish species that are important marine resources, such as *Sparus aurata*. In order to establish laboratory tests and biomarker techniques to assess *in vivo* Cd toxicity in a multilevel (from histological to biochemical) approach, a short-term (48 h) assay was performed using juvenile *S. aurata* injected intraperitoneally with individual Cd dosages (0-8.1 μg Cd $g^{-1}$ fish ww). The results showed that Cd causes a fast and pronounced histopathological degeneration of liver tissue and an exponential induction of liver metallothionein-like proteins (MTs), strongly correlated to the injected Cd dosage (Spearman $R = 0.97$, $p < 0.01$) but not to Cd bioaccumulation or survival time. The relationships between Cd dosage, liver Cd, and liver MT suggested the existence of an absorbed Cd threshold after which the animals were no longer able to regulate and bioaccumulate the metal. This threshold was not dependent on survival time but rather on Cd dose. The findings also confirmed the suitability of *S. aurata* as a test organism regarding toxicity caused by Cd. Complementarily, a histological technique using a fluorochrome (acridine orange) to enhance tissue detail is described, as well as a method suitable for the detection of MTs in SDS-PAGE gels with a colloidal Coomassie blue stain.



### Key-Words

Bioaccumulation; Cadmium; Histopathology; Metallothionein induction, *Sparus aurata*.


### 1. Introduction

Cadmium (Cd) is a non-essential metal that may be found in potentially toxic concentrations in waters and sediments of coastal areas subjected to anthropogenic sources of pollution. Moreover,

[†] Costa & Costa (2008). *Cienc. Mar.* **34**, 349-361 (http://www.cienciasmarinas.com/index.php/cmarinas/article/view/1394).

metal contamination in these areas is affected by physicochemical gradients formed at the sea--freshwater junction (e.g., pH and salinity), which combined with sediment disturbance events may contribute to a release of metals to the water column (Martínez et al., 2006). Cadmium is one of the metals of top concern following point pollution events such as industrial and oil tanker spills. Its presence in the environment and its effects and accumulation in organisms have, for instance, been deeply surveyed in the NW Iberian Peninsula following the Prestige tanker incident (e.g., Férnandez et al., 2006; Prego et al., 2006). Regarding continuous pollution, rivers and estuaries affected by heavy industrial and mining activities are typically problematic areas concerning Cd contamination. Waeles et al. (2004), for instance, estimated that up to 14 kg of dissolved, thus highly bioavailable, Cd are discharged daily from the Loire River (France) to the continental shelf. Audry et al. (2004) found concentrations of dissolved Cd of up to 16.7 $\mu g.L^{-1}$ in another estuarine system in France, even a decade after the shutting down of a mineral ore processing plant that discharged effluents contaminated with Cd. It is noteworthy that the aforesaid concentration is above the chronic toxicity threshold in fish exposed to waterborne Cd (e.g., Chowdhury et al., 2004).

Since Cd is a well-known strong toxicant, considerable research has been performed to assess its bioaccumulation and impact on the health of aquatic organisms, especially concerning important marine resources such as the gilthead seabream, *Sparus aurata* L 1758, a species that represents a valuable catch for coastal fisheries throughout southern Europe and the Mediterranean. This species is often reared in mariculture installations located in estuaries and other enclosed water bodies, potentially of concern in regard to metal contamination. The toxicity of Cd to organisms is responsible for effects at cellular and tissue levels: it is a known genotoxicant, causing both DNA-strand breakage (leading to mutagenesis and carcinogenesis) and chromosomal clastogenesis (originating micronuclei and other nuclear abnormalities; Risso-de Faverney et al., 2001; Costa and Costa, 2007). This metal causes the rupturing of cell and organelle membranes, tissue disaggregation, and neoplasic lesions, and is known to cause abnormal embryogenesis in fish (Jones et al., 2001; Hallare et al., 2005). It triggers a series of different molecular responses, like induction of antioxidative stress enzymes, such as catalase and superoxide dismutase (Žikić et al., 2001), and also induces transcription of glutathione and metallothionein-like proteins that chelate free Cd ions (Singhal et al., 1987; Wormser et al., 1990). The quantification of induction or activity of these responses is frequently employed as a biomarker of Cd contamination.

Metallothioneins (MTs), first described by Margoshes and Vallee (1957) as Cd-binding proteins from horse kidney cortex, are small cytosolic proteins involved in metal storage and detoxification processes. They have about 60 amino acids (none or few of which are aromatic) and a high content of cysteine residues that do not form disulfide bonds and are responsible for sequestering metals. These proteins are very conservative among animal groups and have been widely employed as biomarkers of response to metal exposure, especially to Cd, a known strong MT inducer (reviewed by Romero-Isart and Vašák, 2002), in species including *S. aurata* MT (Tom et al., 1998, 2004; Costa et al., 2008).

Assessment of histopathologies in fish has long been proposed as a sensitive tool for water quality biomonitoring (Handy et al., 2002). Gills and liver are the most surveyed organs in fish due to their role in apical entry and metabolism of contaminants, respectively. In *S. aurata*, gill histopathology has already been employed as a biomarker of exposure to environmental pollution (Morales-Caselles et al., 2007), as well as immunohistochemistry of liver CYP1A regarding exposure to organic contaminants (Arellano et al., 2001). Nevertheless, no specific reference information exists for this species regarding histological liver damage induced by Cd.

Environmental variables such as temperature and pH are known to influence the toxicity of xenobiotics, such as Cd, from biochemical to histopathological endpoints (Carvalho et al., 2004; Hallare et al., 2005). The presence of other contaminants is also known to confound toxicity: Cr, for instance, downregulates MT expression even in the presence of strong MT inducers such as Cd and Zn (Majumder et al., 2003). For these reasons, studies performed on the mechanisms of Cd toxicity under strict laboratory conditions using isolated contaminants and surveying different classes of biomarkers are of great relevance in order to understand the real effects of a specific xenobiotic.

The main goals of the present study were: (i) to test and establish a range of Cd contamination biomarkers and laboratory conditions for *in vivo* assays using *S. aurata* as test organism; (ii) to assess the effects and lethality thresholds of injected Cd (acute and subacute dosages) in this species to serve as reference for future tests; and (iii) to integrate the various effects of contamination, from biochemical (MT induction and Cd accumulation in liver) to histological (in hepatic tissue) levels.

## 2. Material and methods

### *2.1. Laboratory assay*

Juvenile hatchery-brood *S. aurata* from the same cohort (95.3 ± 14.6 mm standard length, 22.5 ± 11.2 g total wet weight [$ww_t$]) were injected intraperitoneally with 500 μL of a Cd standard solution (Merck) diluted in 50 mM phosphate buffer (pH 7.4). Three individuals were tested as controls and injected only with buffer. Six others were injected with several Cd solutions (100-250 μg Cd $L^{-1}$). Together with the animals' different body size, these concentrations allowed different individual Cd dosages to be obtained, within the projected range of 5-10 μg Cd $g^{-1}$ fish $ww_t$. Each animal was subjected to a short-term laboratory assay (48 h) in a 15-L polyvinyl tank, with water recirculation and constant aeration, containing 12 L of the same water in which the fish were reared. Water temperature was held constant at 18 ± 1 ºC, salinity was 31 ± 1, and pH 7.6 ± 0.1. Photoperiod was set at 12:12 h light:dark. Fish were fed commercial fish pellets (Dibaq, Segovia, Spain) daily. The assay was monitored constantly for mortality, and dead or dying individuals were immediately removed from the tanks and processed for bioaccumulation and biomarker analyses.

*2.2. Liver MT and Cd determinations*

Liver portions were homogenized in cold Tris-HCl 0.02 M buffer (pH 8.6) and centrifuged for 1 h at 30,000 g (4 ºC). The supernatant (cytosol) was then heated at 80ºC for 10 min to denaturate non--heat resistant proteins and centrifuged (1 h at 30,000 × g, 4 ºC) to precipitate most of the non-heat resistant and high molecular weight proteins. The determination of MTs from the heat-treated cytosols was performed by differential pulse polarography (DPP), using a static mercury drop electrode (SMDE) with a 694 stand and 693 processor from Metrohm. The electrode system consisted of a mercury capillary working electrode, an Ag/AgCl reference electrode, and a platinum auxiliary electrode. The supporting electrolyte (1 M $NH_4Cl$, 1 M $NH_4OH$, and 2 mM $[Co(NH_3)_6]Cl_3$) was prepared weekly and stored at 4 ºC (Palecek and Pechan 1971). In the absence of a commercial purified fish MT, rabbit MT isoforms 1 and 2 (Sigma) was used for standard addition. The MT extraction and determination by DPP-SMDE followed the procedure described by Costa et al. (2008), adapted from Bebianno and Langston (1989). Results are given in mg MT-equivalent $g^{-1}$ liver dry weight (dw).

The presence of a protein compatible with *S. aurata* MT was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of heat-treated cytosols and rabbit-MT standards. MT is difficult to visualize in gels due to a series of factors such as the near absence of aromatic amino acids, to which Coomassie brilliant blue selectively binds to, and the protein's trend to form polymers in gels (Suzuki et al., 1998), a phenomenon that typically causes a faint protein smear instead of a clear band. An effective method was achieved to detect MT bands without overblotting the cytosolic protein lanes as when silver nitrate is employed. Gels were adapted from Laemmli (1970) and consisted of a 5% acrylamide stacking gel (in 0.5 M Tris-HCl buffer, pH 6.8) and a 15% acrylamide running gel (in 1.5 M Tris-HCl buffer, pH 8.8), both with 10% SDS. Higher percentage of acrylamide (15-19%) enhances resolution of small proteins such as MTs. Aliquots of heat-treated cytosols were further heated at 100ºC during 5 min in sample buffer containing β-mercaptoethanol as reducing agent prior to gel loading in order to promote MT depolymerization. Electrophoresis was run at slow speed (130 V) to promote band resolution, in 3% w/v Tris, 14.4 % w/v glycine, and 1% w/v SDS running buffer. After rinsing with Milli-Q water to wash off all SDS, gels were fixed in 50% v/v methanol and 2% v/v phosphoric acid (85%) until bromphenol blue disappeared, rinsed again, and stained for 24 h by the colloidal Coomassie silver blue method (Candiano et al., 2004). This method provides an intermediate resolution between silver staining and classical colloidal Coomassie brilliant blue staining. The staining solution contained 20% v/v methanol, 10% v/v phosphoric acid (85%), 10% w/v ammonium sulphate, and 0.12% w/v Coomassie brilliant blue G250. The MT molecular weight was determined by comparison of migration distances with a pre-stained broad-range protein ladder (ref. 161-0318, from Bio-Rad), using the Quantity One 4.2.2 software (also from Bio-Rad).

Bioaccumulation of Cd was determined from oven-dried (five days at 80 ºC) liver samples digested with $HNO_3$ and $H_2O_2$ in closed Teflon canisters (Clesceri et al., 1999). The determination of Cd was performed by differential pulse anodic stripping voltammetry (DPASV) with a hanging mercury drop electrode (HMDE), using the aforesaid voltammetric apparatus. The supporting electrolyte consisted of 1 M acetate buffer (pH 4.6). A standard addition method was employed for Cd quantification, using a $CdCl_2$ solution (Merck) as standard. Dogfish liver reference material (DOLT-3, NRC, Canada) was digested using the same procedure and Cd was determined by DPASV-HMDE to control the precision and accuracy of the procedure, and the values obtained were within the certified range. Results are presented in μg Cd $g^{-1}$ liver dry weight (dw).

*2.3. Histological analysis*

Liver portions were fixed in Bouin-Hollande solution for 48 h, washed in distilled water, dehydrated in a progressive series of ethanol, and impregnated with paraffin (xylene was used for intermediate impregnation). Paraffin-embedded sections (2 μm thick) were stained with haematoxylin and eosin (H&E) after deparaffination in xylene and rehydration, and afterwards mounted with DPX resinous medium (from BDH). Sample preparation for histological analyses essentially followed Martoja and Martoja (1967). Other paraffin-embedded sections (2 μm thick) were prepared as abovementioned prior to staining with acridine orange (AO), a fluorochrome that binds selectively to nucleic acids, which was employed to enhance the contrast of cellular structures such as nuclei and endoplasmatic reticuli. The AO staining solution was prepared with 0.1 $g.L^{-1}$ AO base (from Sigma; maximum absorbance 488 nm) in distilled water with 1% v/v acetic acid. Slides were stained with AO solution for 30 min and mounted in DPX after a brief rinse with 0.5% v/v glacial acetic acid in absolute ethanol, followed by 1 min in absolute ethanol and 1 min in xylene. All steps from staining onwards (inclusively) were performed using opaque Hellendahl jars to prevent the loss of fluorescence.

Slides were qualitatively analyzed for hepatic lesions with bright-field illumination for H&E staining and with ultraviolet light for AO staining, using a BX50 microscope adapted for epifluorescence with a U-ULH 100 W mercury high-pressure bulb and a U-MWB fluorescein filter (all equipment from Olympus).

*2.4. Statistical analyses*

Data were analyzed using linear regression models, employing the $r^2$ statistic to assess goodness-of-fit of graphical functions, and by Spearman's rank-order correlation $R$ statistic. Regressions where $r^2 < 0.5$ were disregarded and the significance level for correlations was set at $\alpha = 0.05$. Statistics were performed with Statistica (Statsoft Inc.), after Sheskin (2000).

## 3. Results

### *3.1. Lethality thresholds, MT induction, and Cd accumulation*

All control individuals survived the assay but only two animals injected with Cd survived the entire duration of the assay. Despite one individual having survived a dosage of 8.1 µg Cd $g^{-1}$ fish ww, it is apparent that a possible survival time threshold for at least 48 h after injection may be set at dosages between 4 and 5 µg Cd $g^{-1}$ fish ww. A better fit regression model was found between liver MT concentration and injected Cd dosages than between liver MT and Liver Cd. The first case showed the existence of a strong exponential relationship between liver MT and injected Cd dosages (Fig. 2.2.1a), whereas MT × liver Cd (Fig. 2.2.1b) fitted better to a negative second order polynomial model. No significant regression ($r^2 < 0.5$) was found regarding liver Cd × Cd dosage. This showed that liver Cd levels did not increase with dosage and were too variable to be able to obtain a clear trend. In accordance with the regression results, a strong correlation was found between injected Cd dosages and liver MT (Spearman $R = 0.97$, $p < 0.01$). Conversely, no significant correlation was observed between MT and liver Cd ($p > 0.05$), which is in accordance with the fact that liver MT did not increase at higher levels of bioaccumulated Cd. No significant correlations were found between the injected Cd dosages and liver Cd, or between survival time and the variables: liver MT, accumulated Cd, and injected Cd dosage ($p > 0.05$).

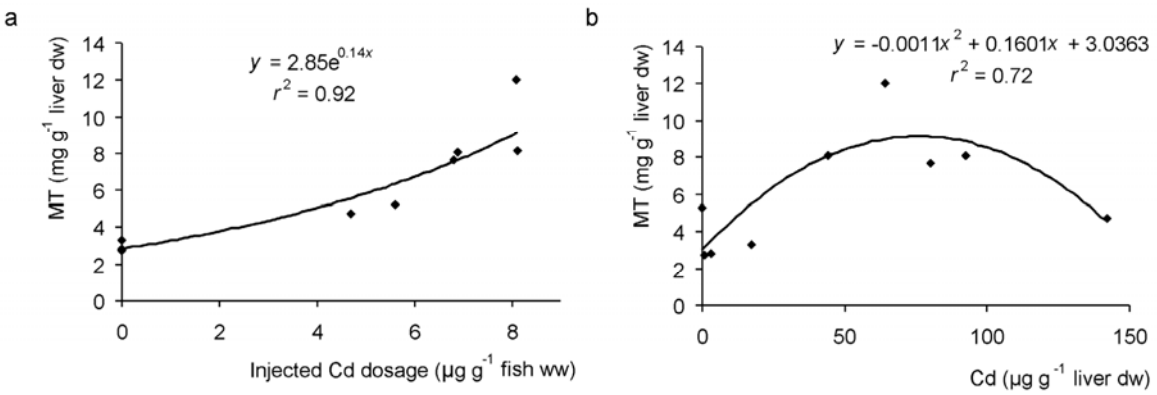


**Fig. 2.2.1.** Best fit regression models between significantly correlated variables: (a) liver MT × injected Cd dosage, and (b) liver MT × liver Cd.

The MT × liver Cd model showed an inflexion point that marked the threshold after which the liver does not seem capable of regulating Cd by inducing MT biosynthesis. Regarding MT induction, there was a notorious decrease in liver MT synthesis after the Cd accumulation threshold value of ≈ 80 µg.$g^{-1}$ liver dw.

The SDS-PAGE gels showed a dim but consistent band in heat-treated cytosol samples, with approximately the same molecular weight as the rabbit liver MT used as standard that may be assigned

to *S. aurata* MT (Fig. 2.2.2). The estimated molecular weight for the dimeric form of MT was 15 ± 1 kDa for both rabbit standard and *S. aurata* MT.

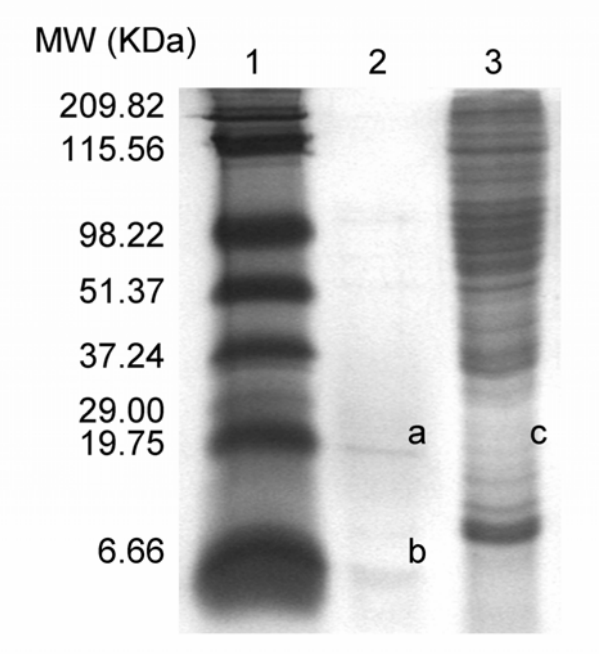


**Fig. 2.2.2.** SDS-PAGE gel of heat-treated cytosol: lane 1 = broad-range MW standard, lane 2 = rabbit MT standard, and lane 3 = heat-treated *Sparus aurata* cytosol (from a control individual); a = dimeric form of rabbit MT (15 ± 1 kDa), b = monomeric form of rabbit MT (6 ± 1 kDa) and c = dimeric form of *S. aurata* MT (15 ± 1 kDa).

*3.2. Histopathology*

Control individuals showed a normal structure of hepatic tissue: hepatocytes were well individualized and polyedric in shape, with conspicuous constant-sized nuclei with individualized nucleoli. The liver exhibited inclusions of pancreatic tissue surrounding the branches of the hepatic portal vein and many sinusoids (Fig. 2.2.3).

The Cd-injected individuals exhibited a consistent and very pronounced degradation of hepatic tissue that increased progressively with Cd dosage, regardless of survival time (Fig. 2.2.4). Hepatocyte degeneration was the most evident damage, depicting loss of cell shape and plasmatic membrane rupture. Liver sections also presented large areas of necrosis, where tissue organization was no longer perceptible, especially in individuals injected with the highest Cd dosages (> 6 μg Cd $g^{-1}$ fish $ww_t$). Pancreatic tissue was strongly stained by AO, as expected, since the fluorochrome binds especially to nucleic acids and therefore the dense rough endoplasmatic reticulum typical of high enzyme-secretion cells (such as the acinar cells of the pancreas) emits a strong fluorescence. A prominent degeneration, more conspicuous with AO staining, of this tissue was noticeable in Cd-injected individuals, reaching the status of complete necrosis of the pancreatic tissue at higher dosages of Cd. Fluorescence

microscopy using AO staining provided an overall better resolution of nuclei and cellular membranes than HE, especially at higher magnifications, and thus provided enhanced detail of hepatic damage.

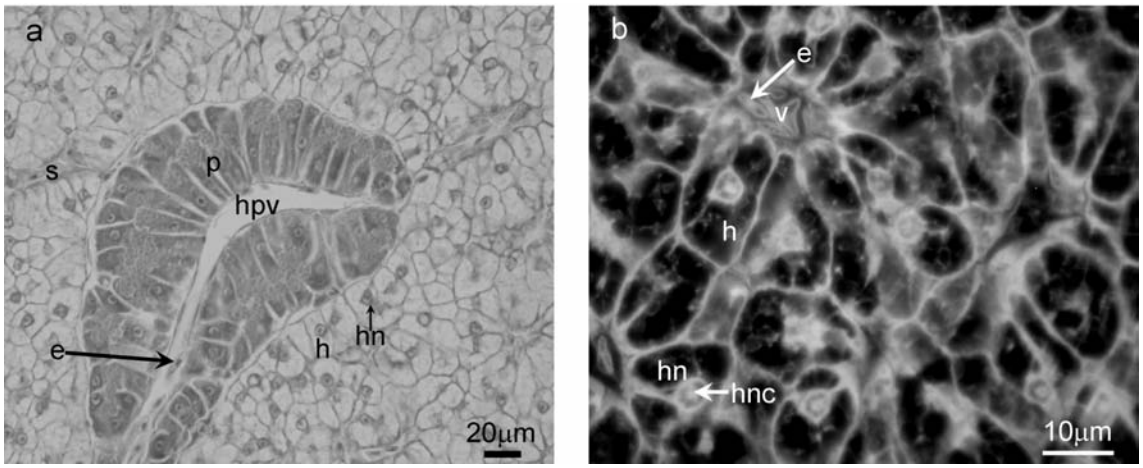


**Fig. 2.2.3.** Normal histological structure of the liver of *Sparus aurata* (control individual): (a) haematoxylin--eosin staining (× 400), and (b) acridine orange staining (× 1,000). Erythrocyte in blood vessel (e), hepatocyte (h), hepatocyte nucleus (hn), hepatocyte nucleolus (hnc), hepatic portal vein branch (hpv), pancreatic tissue (p), sinusoid (s), and venule (v).

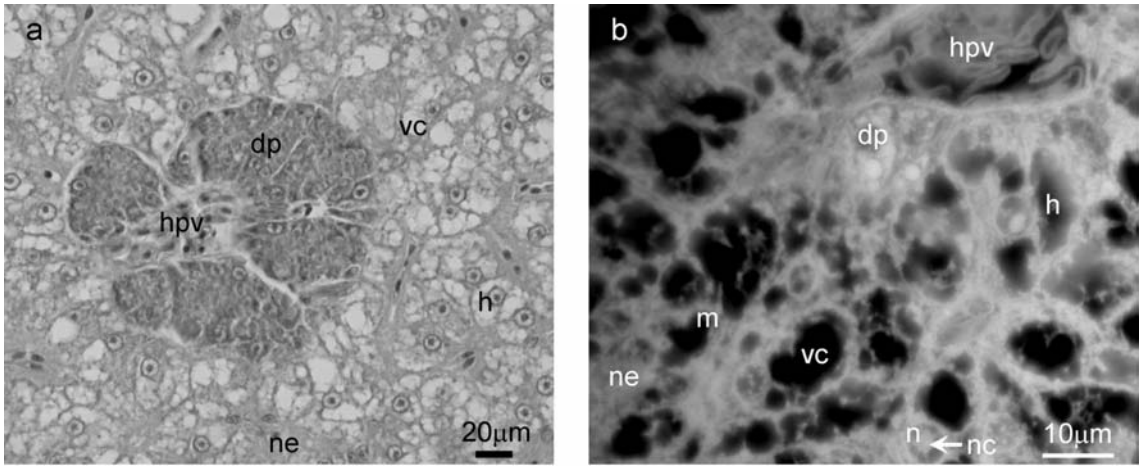


**Fig. 2.2.4.** Degenerating hepatic tissue of *Sparus aurata* (Cd-injected individual surviving after 48 h): (a) haematoxylin-eosin staining (× 400), and (b) acridine orange staining (× 1,000). Degenerative pancreatic tissue (dp), hepatocyte (h), hepatic portal vein branch (hpv), rupturing cellular membrane (m), hepatocyte nucleus (n), hepatocyte nucleolus (hnc), necrotic tissue (ne), and vacuole resulting from fatty degeneration (lipidosis) (vc).

No differences were observed regarding sinusoid number and structure between control and Cd-injected individuals. Overall structural analysis revealed a vacuolated aspect of Cd injected animals most likely due to an increase in intracellular storage of lipids (termed as fatty degeneration or hepatocellular lipidosis). This resulted in the proliferation of large vacuolar structures or aggregates of smaller vacuoles in hepatocytes (Fig. 2.2.5). This finding is in accordance with previous studies that described hepatic vacuolation as a non-specific alteration occurring in fish chronically exposed to waterborne metals (e.g., Arellano et al., 1999).

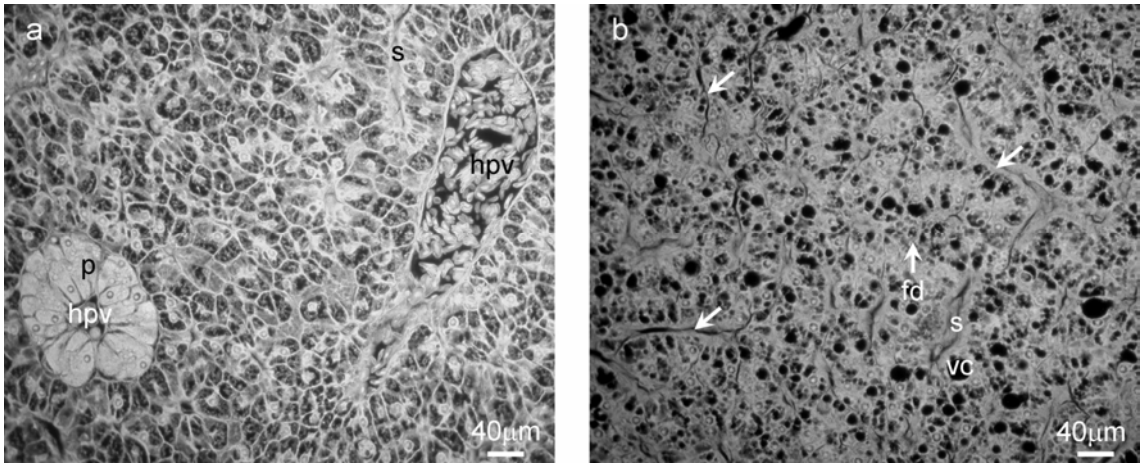


**Fig. 2.2.5.** General structure of *Sparus aurata* liver (acridine orange, × 400): (a) normal hepatic tissue from a control individual and (b) damaged liver from a Cd-injected individual (survival time < 24 h). Fatty degeneration (lipidosis) exhibited as an aggregate of small vacuoles (fd), hepatic portal vein branch (hpv), sinusoid (s), normal pancreatic tissue exhibiting the highly fluorescent cytoplasm of acinar cells (p), and fatty degeneration demonstrated as a large vacuole (vc). Arrows indicate structural splitting of tissue.

## 4. Discussion

In this study we have demonstrated that Cd accumulation and MT induction in fish liver are not linearly correlated. Instead, they reflect the metabolic capability of the organ, which is progressively affected by metal dosage, as shown by the histopathological evaluation. Both liver MT and Cd show a decrease from the threshold point where the liver fails to detoxify/accumulate the metal and begins a degeneration process that ultimately leads to organ failure and animal death. According to the present results, the time elapsed after Cd injection does not seem to be a crucial factor regarding the magnitude of responses and effects, unlike Cd dosage. This seems to be a sensible finding since Cd injected intraperitoneally is likely to be quickly absorbed by nearby organs and thus made readily available to affect tissues and cells. In fact, fast Cd absorption and intracellular mobilization have been found in liver cell cultures and single-cell organisms incubated in Cd solutions (Diep et al., 2005; Arunakumara et al., 2007). Conversely, exposure assays to waterborne metals depict pronounced time--dependent levels of effects and responses (Van Dyk et al., 2007), as opposed to the more dose--dependent observed in this study. It may therefore be concluded that Cd is a fast-acting toxicant once it has entered the organism and is absorbed by organs.

Regarding direct responses to intoxication, MT induction is independent of both the survival time elapsed after injection and liver Cd, but it is strongly correlated to Cd dosage, suggesting that MT biosynthesis in liver is rapidly induced by the presence of Cd but is not necessarily linked to bioaccumulated Cd in the organ. The low correlation between MT induction and accumulated Cd is similar to data obtained from cultured hepatocytes exposed to the metal and is yet to be explained

(Diep et al., 2005). Nevertheless, the Cd-injected individuals surviving less than 24 h on average induced MT at levels 2.5-fold higher (7.2 ± 1.4 $mg.g^{-1}$ liver dw) than the control individuals (3.0 ± 0.3 $mg.g^{-1}$ liver dw). The fish surviving after 48 h induced MT 1.6- and 4.1-fold, corresponding to Cd dosages of 4.7 and 8.1 μg Cd $g^{-1}$ fish ww, respectively. These results are in accordance with the fast induction of other proteins involved in detoxification processes. Vaglio and Landriscina (1999), for instance, described an induction of 25% and 40% of CYP1A-related proteins in *S. aurata* injected with 2.5 μg Cd $g^{-1}$ fish ww after just three and four days of Cd injection, respectively, in a similar *in vivo* test.

Considering the severity of the hepatic lesions observed, especially irreversible damage such as necrosis, it should not be disregarded that liver health may compromise MT induction and metal bioaccumulation. This may explain why at higher dosages of injected Cd the protein was not induced and the animals were not able to accumulate the metal, since hepatic tissue sustained great damage. Organ integrity may be a very important factor that contributes to the typical wide intrapopulation variability observed for MT induction in previous studies (e.g., Mouneyrac et al., 2002; Costa et al., 2008). This issue should be addressed, as should other biological variables, such as age and sex, known to contribute to the variability of MT induction (Hamza-Chaffai et al., 1995), for instance by surveying the population baseline liver lesions.

The present findings indicate that MT induction in the liver may be positively linked to the extent of hepatic lesions. This may be explained by the known link between antioxidant responses and MT induction, since it is likely that liver tissue degeneration produces oxidative radicals (Risso-de Faverney et al., 2001). Consequently, antioxidant mechanisms may have been triggered by functional hepatocytes, further enhancing MT transcription (Bi et al., 2004). On the other hand, Cd bioaccumulation appears to be impaired by the severity of lesions observed in fish injected with the highest Cd dosages. This may contribute to an overall reduction in the survival time after Cd injection since accumulated Cd may be less available to affect cellular functions.

Regarding SDS-PAGE analysis, the estimated molecular weight of *S. aurata* MT is consistent for dimeric forms of the typically ≈ 6-7 kDa vertebrate MTs, a range in which *S. aurata* MT is included (Tom et al., 1998). The present method successfully allowed MT to be visualized without overstaining of the wide molecular-weight range peptides found in heat-treated cytosols, in spite of the low MT concentrations in cytosolic samples that resulted in dim bands. The presence of unidentified peptides in cytosolic extracts has been observed in similar studies on marine organisms that used the same extraction method (e.g., Bustamante et al., 2002; Correia et al., 2004), but these are not metal--induced and should thus not interfere with the determination of MT induction (Bebianno et al., 1992).

Our results successfully provided an insight of the mechanistics of Cd toxicity at tissue and biochemical levels. It should be noted that toxicological studies on aquatic organisms involving environmental contaminants in water or in sediments are typically constrained by variables such as xenobiotic bioavailability and interactions, and the characteristics of the mechanisms of apical entry and detoxification. Altogether, these are confounding factors that may impair the evaluation of the

actual toxicity effects and responses, for example, by providing biomarker and bioaccumulation evaluations with a difficult to explain variability. *In vivo* studies such as the present experiment allow a more objective interpretation of the toxicological effects of contaminants, which is a crucial part of posterior studies involving environmental xenobiotics tested under laboratory or even field conditions. Our research has established test and biomarker techniques for *S. aurata* and provides reference information on the biomarkers studied.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and the POCTI research project (Programa Operacional Ciência, Tecnologia e Inovação) ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). The first author was supported by a FCT PhD. grant (SFRH/BD/28465/2006). Special thanks to M. Diniz (REQUIMTE) and J. Inácio (DCV) for their support and also to Timar/Ria Mãe (Setúbal, Portugal) for supplying the fish used in the experiment.

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# Chapter 3. Laboratory assays

## 3.1. Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants[†]


### Abstract

Juvenile *Solea senegalensis* (Senegalese sole) were exposed to freshly collected sediments from three sites of the Sado Estuary (West-Portuguese coast) in 28-day laboratory assays in order to assess the ecological risk from sediment contaminants, by measuring two genotoxicity biomarkers in peripheral blood: the percentage of Erythrocyte Nuclear Abnormalities (ENA) by use of an adaptation of the micronucleus test, and the percentage of DNA strand-breakage (DNA-SB) with the comet assay. Sediments were surveyed for metallic (Cr, Ni, Cu, Zn, As, Cd and Pb) and organic [PAHs (polycyclic aromatic hydrocarbons), PCBs (polychlorinated biphenyls) and DDTs (dichloro-diphenyl--trichloroethane)] contaminants. Sediments from site A (farthest from hotspots of contamination) were found to be the least contaminated and weaker inducers of genotoxic damage, whereas sediments from sites B (urban influence) and C (affected by industrial effluents and agricultural runoffs) were responsible for a very significant increase in both ENA and DNA-SB, site B being most contaminated with metals and site C mainly with organic pollutants, especially PAHs and PCBs. Analysis of genotoxic effects showed a strong correlation between the concentrations of PAHs and PCBs and both biomarkers at sampling times $T_{14}$ and $T_{28}$, while the amounts of Cu, As, Cd and Pb were less strongly correlated, and at $T_{28}$ only, with ENA and DNA-SB. These results show that organic contaminants in sediment are stronger and faster acting genotoxic stressors. The results also suggest that metals may have an inhibitory effect on genotoxicity when interacting with organic contaminants, at least during early exposure. ENA and DNA-SB do not show a linear relationship, but a strong correlation exists between the overall increase in genotoxicity caused by exposure to sediment, confirming that they are different, and possibly non-linked effects that respond similarly to exposure. Although the comet assay showed enhanced sensitivity, the two analyses are complementary and suitable for the biomonitoring of sediment contaminants in a benthic species like *S. senegalensis*.



### Keywords

Genotoxicity; Erythrocyte nuclear abnormalities; Comet assay; *Solea senegalensis*; Contaminated sediments; Sado Estuary


[†] Costa et al. (2008). *Mutat. Res.* **654**, 29-37 (doi:10.1016/j.mrgentox.2008.04.007).

## 1. Introduction

The presence of DNA and chromosome damage and the efficacy of damage repair have gained growing concern regarding toxic substances, since they are not only directly linked to cell survival but also to mutagenesis and carcinogenesis. Many environmental contaminants are known to induce damage to chromosomes and DNA, and genotoxicity has been integrated in biomonitoring programs to assess exposure to xenobiotics. Much research is now being focused on human populations exposed to contaminants [e.g., workers in health-hazardous industries (Sul et al., 2003; Sailaja et al., 2006)] and to animal species living in potentially contaminated environments. For such purposes, a series of genotoxicity assessment assays has been developed and is frequently applied and put to test in a wide range of organisms, from the classic micronucleus (MN) test to the agarose gel DNA strand breakage assay, the single-cell gel electrophoresis (comet) assay and the analysis of xenobiotics-DNA adducts. The employment of these techniques has become widespread among toxicologists involved in research at different levels: from biomedicine to environmental sciences.

The analysis of erythrocyte nuclear abnormalities (ENA) comprises a variant of the standard micronucleus test and is widely used in fish toxicology. In this assay, a number of alterations in cell nuclei that may lead to their fragmentation and/or to micronucleus formation are recorded instead of counting the micronuclei themselves, which are rare and frequently measured by subjective scoring (Ayllon and García-Vázquez, 2000; Çavas and Ergene-Gözükara, 2005; Ergene et al., 2007; Costa and Costa, 2007). Although originally applied to human health issues, ENA analysis and the MN test have become rather common as simple tests for genotoxicity of organic and metallic contaminants in fish, since fish erythrocytes are nucleated (Ayllon and García-Vázquez, 2000; Çavas et al., 2005; Baršienė et al., 2006). There are many other classical tests in fish blood cells that have become widespread due to their simplicity and feasibility, such as analyses of membrane permeability and cell shape (Nikinmaa, 1992).

The alkaline version of the comet assay has become a common technique for detection of DNA damage (chain-fragmentation) resulting from the combination of single- and double-strand breaks as well as alkali-labile sites (formed by excision of damaged nucleobases) and xenobiotic-DNA adducts that break during electrophoresis (Singh et al., 1988). The comet assay has been proposed as an effective tool for biomonitoring organic contaminants like pesticides and their derivatives (Blasiak and Trzeciak, 1998), metals such as cadmium (Desai et al., 2006) and for the monitoring of waters contaminated with complex mixtures (Alink et al., 2007).

The employment of both ENA and the comet assay for genotoxicity assessment in fish has been proposed before, since they are related to different levels of degradation of genetic material: ENAs reflect chromosome-level genotoxicity, whereas the alkaline comet assay allows assessment of DNA damage at the molecular level (Wirzinger et al., 2007) through quantification of total strand--breaks. It is noteworthy, though, that genotoxicity assays have mainly focused on *in vitro* tests for the effects of isolated contaminants in a specific cell population (e.g., fish hepatocytes and mammalian

lymphocytes), or *in vivo* assays of waterborne isolated or (few) mixed contaminants. Recently, research has begun to focus on genotoxic effects in field collected aquatic animals and on assays with sediments using fish and aquatic invertebrates. Nevertheless, most of these studies deal with one or a limited number of contaminants and thus research is still missing concerning the relative potency of different types of contaminant in inducing damage in chromosomes or DNA, and concerning the genotoxic effects of a complex mixture of different kinds of contaminant, such as in sediments in general and in estuarine sediments in particular.

The Sado Estuary is one of the largest estuarine areas in Europe. It is subject to a large variety of anthropogenic usages and alterations that often collide: while a large part of the estuary is classified as a natural reserve and the Tróia Peninsula is an important tourism and leisure area, the city of Setúbal has one of the largest heavy-industry concentrations in Portugal, including mineral-ore deployment facilities, chemical plants, paper mills, shipyards and a large thermoelectrical unit. The estuary is also very important for fisheries and aquaculture, which together represent a large portion of the local society's income and economy. For these reasons, efforts have been made to establish risk assessment strategies in the estuary, which involve analysis of contaminants (Caeiro et al., 2005) and biomarker approaches using bioassays (Costa et al., 2008).

*Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae) is a very common benthic fish species in the Sado Estuary where, together with other flatfish, it is a regular target or at least a valuable by-catch for local fisheries. Reproductive adults enter the estuary for reproduction in the summer, and as a consequence, the population reaches the highest number of individuals in the autumn (Cabral, 2000b). This species inhabits sandy or muddy bottoms and scavenges the sediment for feeding on small benthic invertebrates like polychaetes, amphipods and bivalves (Cabral, 2000a; Sá et al., 2003). *S. senegalensis* may be exposed to sediment contaminants by foraging on benthic fauna and also by direct contact (e.g., through gill epithelia) with sediment particles or interstitial water. The benthic nature of the species and the fact that it is a very common species along the Atlantic coasts of the Iberian Peninsula render *S. senegalensis* particularly interesting with respect to bio-monitoring of sediment contaminants in the Peninsula (Riba et al., 2004).

The main goals of the present work are (i) assessment of genotoxicity biomarkers in a benthic fish as an effective tool for biomonitoring sediment contaminants, (ii) to compare the relative potency of sediment metallic and organic contaminants in terms of genotoxic effects, and (iii) to compare two different genotoxicity indicators: ENA and total DNA strand breakage (DNA-SB).

## 2. Methods and Materials

### *2.1. Experimental assay*

The sediments to be tested were collected from three sites of the Sado Estuary (Fig. 3.1.1).

Station A is located near an environmentally protected area, and is the farthest from direct contamination sources. Due to its geographical location in the estuary, this site has comparatively stronger influence from ocean hydrodynamics and shorter water residence time than the other sites. Site B, near the port of Setúbal, and site C, just off the city's heavy-industry area, were surveyed as potentially contaminated. They are located in an area of lower hydrodynamics and stronger river influence, which facilitates retention of contaminants and smaller sediment particles.

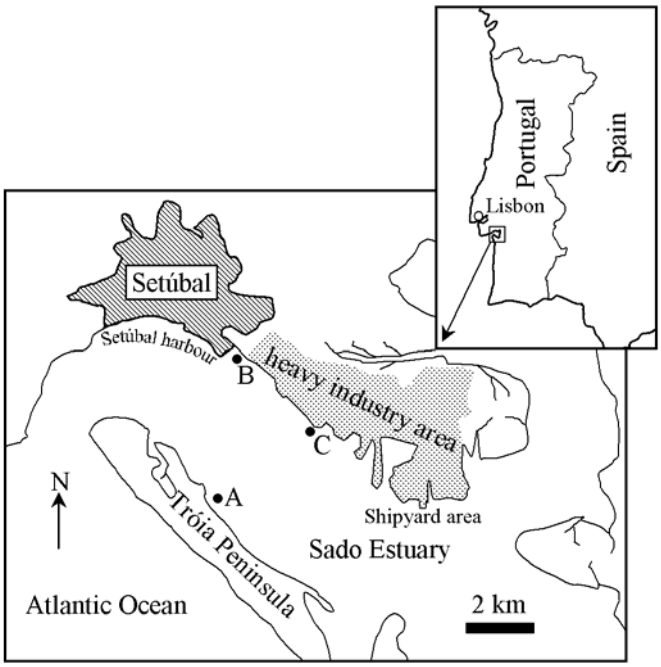


**Fig. 3.1.1.** Map of the study area with sediment collection sites (•).

Sediment collection was made near the shore, at a maximum depth of 9 m, in November 2006. Freshly collected sediments were used in the assays and homogenized portions were immediately frozen for subsequent analyses.

Juvenile hatchery-brood and laboratory-reared *S. senegalensis* belonging to the same cohort (69 ± 6 mm standard length), obtained from aquaculture research facilities (IPIMAR/CripSul-Estação Piloto de Piscicultura, Olhão, Portugal), were randomly distributed in aerated tanks (capacity, 15 L) each containing 2 L of sediment (525 $cm^2$ of sediment surface) and 12 L of water, with 24 animals being allocated per tank. Sediments were allowed to settle for 48 h prior to the beginning of the assay. The 28-day dynamic, assays were performed in duplicate, with three sampling times: day 0 ($T_0$), day 14 ($T_{14}$) and day 28 ($T_{28}$). Fish were fed daily with Aquasoja M2 commercial fish pellets (Sorgal, Ovar, Portugal).Water parameters (pH 7.9 ± 0.2, salinity = 33 ± 1, temperature = 18 ± 1 ºC, $DO_2$ = 40--45%, and total ammonia = 2-4 $mg.L^{-1}$) were monitored and held constant for all tests through weekly water changes (25% of the total volume) in order to mimic rearing conditions.

*2.2. Sediment analysis*

Sediments were surveyed for fine fraction (FF), total organic matter (TOM) and redox potential (Eh) since genotoxicity may be influenced not only by the contaminants' concentration but also by sediment parameters influencing their bioavailability. FF (particle size < 63 μm) was obtained by removal of organic matter with $H_2O_2$, followed by disaggregation with pyrophosphate, washing and hydraulic sieving. Results are expressed as percentage FF of the total dry weight, dw) of the sediment. Sediment TOM was quantified by total ignition at 500 ± 25 ºC for 4 h. Results are expressed as TOM percentage relatively to sediment dw. Sediment Eh was measured immediately following collection, using an Orion model 20A meter with a H3131 Ag/AgCl reference electrode (Orion Research Inc.). Sediment samples were oven-dried to constant weight prior to determination of TOM, FF and contaminant concentrations in order to express results relatively to sediment dry weight (dw).

Sediments were surveyed for the metals chromium (Cr), nickel (Ni), copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn) and the metalloid arsenic (As). Approximately 100 mg of sediment was completely mineralized with 6mL HF (40%, v/v) and 1 mL of Aqua-Regia (36% HCl and 60% $HNO_3$; 3:1 v/v) in closed Teflon vessels at 100 ºC during 1 h. The contents were evaporated to near dryness in Teflon vials, redissolved in $HNO_3$, heated for 20 min at 75 ºC and diluted to 50 mL with Milli-Q water (Caetano et al., 2007). Concentrations of Cr, Ni, Cu, Zn, As, Cd and Pb were determined using a quadropole inductively coupled plasma mass spectrometer (ICP-MS) (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. A seven--point calibration curve in the range between 1 and 100 $\mu g.L^{-1}$ was used to quantify metal concentrations. Variation coefficients for metal readings ranged between 0.5 and 2%. Method accuracy was determined by analysis of procedural blanks and sediment reference materials: MESS-2 (NRC, Canada), PACS-2 (NRC, Canada) and MAG-1 (USGS, USA) and metal concentrations were found within the certified range. Results are given in $mg.kg^{-1}$ sediment dw.

PAHs (polycyclic aromatic hydrocarbons) were analysed from dried sediments spiked with 1 mL surrogate standards (Supelco) containing acenaphthene-d10 (0.408 $\mu g.mL^{-1}$), pyrene-d10 (0.397 $\mu g.mL^{-1}$), chrysene-d12 (0.397 $\mu g.mL^{-1}$) and perylene-d12 (0.433 $\mu g.mL^{-1}$) and Soxhlet-extracted with 250 mL of a mixture of acetone and hexane (1:1, v/v) for 36 h (Martins et al., 2008). The extract was concentrated by evaporation and fractionated with a silica-alumina (1:1) column. PAH compounds were then eluted with hexane/dichloromethane (9:1 and 4:1, v/v) and evaporated. Determination of PAHs was performed on a GCQtrace Finnigan gas chromatography-mass spectrometry (GC-MS) system in selected ion monitoring (SIM) mode. An autosampler and DB-5 column (30 m, 0.25 mm, 0.25 μm) were used. Identification of PAH compounds was based on the comparison of their GC-retention times and their mass spectrum, with appropriate individual standards. Concentrations of individual PAHs were measured by the internal standard peaks area method and a nine-point calibration curve for each compound. The detection limit was calculated with a signal-to-noise ratio of 3:1 in a blank sample ($n$ = 5) and varied within a narrow interval around

0.001 μg.g$^{-1}$. Results are expressed in ng.g$^{-1}$ sediment dw. Total PAH (tPAH) means the sum of all analysed compounds.

PCB (polychlorinated biphenyls) and DDT (dichloro-diphenyl-trichloroethane) determinations in sediments were carried out in dry sediment samples that had been Soxhlet-extracted with n-hexane for 16 h (Ferreira et al., 2003). The extracts were fractionated with a Florisil chromatographic column. PCBs and *pp*'DDE were eluted with 15 mL of n-hexane and *pp*'DDD and *pp*'DDT were eluted with 45mL of dichloromethane/hexane (30:70, v/v). The extracts were purified with sulphuric acid and the elemental sulphur was removed with activated copper. PCB congeners and *pp*'DDT plus metabolites (*pp*'DDD and *pp*'DDE) were analysed using a Hewlett-Packard 6890 gas chromatograph with an electron-capture detector and a capillary column (DB5, J&W, 60 m). Quantification was obtained by the external standard method, using a seven-point calibration curve for each compound. In the present study tPCB means the sum of congeners with IUPAC numbers and tDDT means the sum of *pp*'DDT plus the *pp*'DDD and *pp*'DDE metabolites. Concentrations are given in ng.g$^{-1}$ sediment dw. The analytical detection limit was 0.01 ng.g$^{-1}$.

Quality control for organic contaminants was obtained by analysis of the certified sediment SRM 1941b (NIST, USA) and blanks, included in each batch of 12 samples. Recoveries of analysed PAHs in the certified material ranged from 73 to 112%, and from 93 to 109.2% for PCBs and DDTs.

*2.3. Genotoxicity assessment*

Genotoxic effects were assessed by the alkaline version of the single-cell gel electrophoresis ("comet") assay and ENA analysis in fish peripheral blood. Blood was collected from twelve animals per test and per sampling time (six per replicate) just above the lateral line system with a syringe previously washed with 0.1M EDTA to prevent clotting. Blood aliquots were immediately smeared on glass microscopy slides for ENA analysis and diluted in cold 50 mM PBS (phosphate-buffered saline) with 0.7% NaCl, pH 7.4 (dilution factor: 200) for the comet assay, which was performed right after blood collection to ensure maximum cell viability.

ENA analysis was performed by staining methanol-fixed (15 min) blood smears with 0.1 g.L$^{-1}$ acridine orange (Sigma; maximum absorbance at 488 nm). Slides were afterwards mounted with DPX (from BDH). The procedure follows Costa and Costa (2007). About 1,000 mature erythrocytes per slide were observed in order to determine the percentage of cells with nuclear abnormalities. The ENA considered were: micronuclei, nuclear buds, polynucleated cells and fragmenting nuclei (Costa and Costa, 2007; Fenech et al., 2003). Results are expressed as the percentage of mature erythrocytes showing nuclear abnormalities.

The neutral comet assay was performed as described by Singh et al. (1988). In brief: 10 μL of cell suspension in PBS was diluted in 180 μL of liquid (35 - 40 ºC) 1% (w/v) low melting-point agarose (LMPA; Sigma) dissolved in PBS. Aliquots (2 × 75 μL) of cell-containing LMPA were placed on slides pre-coated with 1% (w/v) normal melting-point agarose in TAE buffer (dried for at least 24

h) and covered with a coverslip. After agarose solidification (15 min, 4 ºC) the coverslip was removed and the slides were dipped for 1 h at 4 ºC in lysis solution (2.64% NaCl (w/v), 3.72% EDTA (w/v) and 5 mM TRIS) to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. Slides were afterwards placed in cold (4 ºC) electrophoresis solution (pH 13) for 40 min to allow DNA unwinding and enhanced expression of alkali-labile sites. Electrophoresis was run for 30 min at 25 V, in the cold (4 ºC), using a Sub-Cell model 96 apparatus (Bio-Rad). Slides were afterwards neutralized in 0.1 M Tris-HCl buffer (pH 7.5) for 15 min. All preparatory steps were performed under controlled temperature (≈ 16 ºC) to avoid gel lifting from the slides and all solutions and electrophoresis apparatus were kept in the dark and in the cold to minimize accessory DNA degradation. Approximately 100 comets were analysed per slide after staining with 0.02 $mg.mL^{-1}$ ethidium bromide. Comets were analysed using the software CometScore 1.5 (TriTek Corp.). The percentage DNA in the tail - one of the most consensual comet metrics -was employed as a direct measure of DNA-SB (Lee and Steinert, 2003). Results are expressed in average percentage of total DNA-SB per individual. Erythrocyte vitality was assessed by use of the propidium iodide test, a fluorochrome that penetrates only dead or damaged cells, and at least 70% viable cells were observed.

A DMLB microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps was used for both analyses, equipped with an I3 filter (used for acridine orange staining) and a N2.1 filter (used for ethidium bromide staining), all from Leica Microsystems.

### *2.4. Statistical analysis*

Data were analysed by non-parametric statistics after invalidation of variances' homogeneity of ENA and comet assay data by the Levene's test. Analysis of variance was performed using the Kruskall-Wallis ANOVA by ranks $H$ statistic for overall differences between tests, the Mann-Whitney $U$ test for pairwise comparisons within tests and sampling times and the Spearman rank-order correlation $R$ statistic for sediment contaminants and genotoxicity analyses. The significance level for all analyses was set at $\alpha = 0.05$. All statistics were performed with the software Statistica (Statsoft Inc.).

## 3. Results

Overall mortality at the end of the assay was very different between tests: 2% for sediment A, 13% for B and 48% for C. Replicate effect between tests was found to be non-significant for both ENA and DNA-SB analyses, $p = 0.67$ and $p = 0.43$, respectively (Kruskall-Wallis $H$). Fish standard length and total fresh weight ranges were 70 ± 6 mm and 4.21 ± 1.53 g, respectively, at the end of the assays and no significant differences were found between tests and sampling times regarding both measures (Kruskall-Wallis $H$, $p > 0.05$).

*3.1. Sediment parameters and contaminants*

The sediments from the three sites revealed distinct characteristics (Table 3.1.1) and different levels of contamination regarding metallic (Fig. 3.1.2) and organic (Fig. 3.1.3) contaminants. Sediment A is the least contaminated by organic substances and shows the lowest levels of Cd, As, Cu and Pb. The sediment from this site is essentially sandy, with little organic matter, while sediments B and C present high FF and TOM and lower Eh. Sediment B is the most contaminated with each of the metals studied and also presents high levels of PAHs. Sediment C is essentially contaminated by organic compounds, especially PAHs and PCBs. The main contributions in the PAH contamination in sediments are from 4- and 5-ring PAHs, which in combination represent 73.8% of tPAH in sediment A, 70.6% in B and 67.7% in C. Penta- and hexa-chlorinated compounds are the most representative PCBs, especially in sediment C (91.1% relatively to tPCB). DDT contamination was found to be low at all sites, the main contribution coming from *pp*'DDT (82.4% in sediment A, 88.9% in B and 48.5% in C, relatively to tDDT) (Table 3.1.2).

**Table 3.1.1**. Characterization of the tested sediments.

| Site | **FF (%)** | **TOM (%)** | **Corrected Eh (mV)** |
|---|---|---|---|
| A | 37.3 | 3.2 | -233 |
| B | 97.9 | 11.8 | -290 |
| C | 76.8 | 7.7 | -316 |

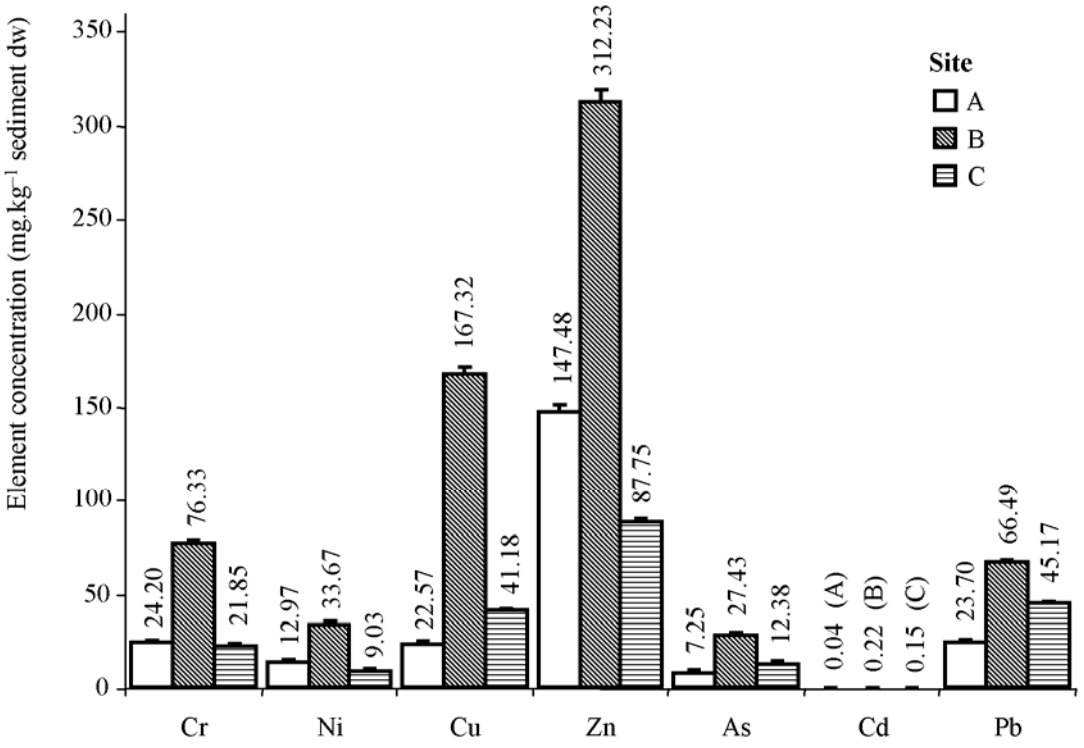


**Fig. 3.1.2.** Concentration of Cr, Ni, Cu, Zn, As, Cd and Pb in tested sediments. Error bars indicate the standard error.

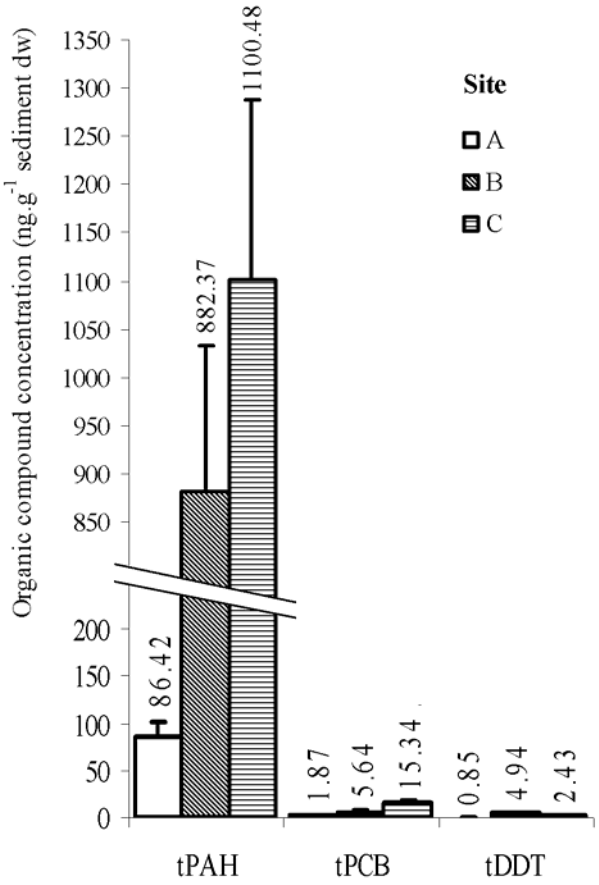


**Fig. 3.1.3.** Concentration of organic contaminants in sediments. Error bars indicate the standard error.

*3.2. Genotoxicity*

Nuclear buds, lobed nuclei or a combination of these two alterations (Fig. 3.1.4) comprised the large majority of ENA observed, with an average of 74.1 ± 23.8% of total observed ENA in the most affected individuals (%ENA > 16%). The observed nuclear alterations are in accordance with previous studies using acridine-orange staining (Costa and Costa, 2007). A significant increment in average percentage of ENA was observed for all stations at $T_{28}$, relative to $T_0$, but only significant at $T_{14}$ regarding the test with sediment C (Fig. 3.1.5). From$T_{14}$ to $T_{28}$ tests A and B revealed a significant increase in ENA (Mann-Whitney *U* test, $p < 0.05$ and $p < 0.01$, respectively), whereas test C showed a less significant increment in average percentage of ENA (Mann-Whitney *U* test, $p = 0.12$).

A similar pattern to that obtained from ENA analysis was seen in the comet assay (Fig. 3.1.6) results but differences between tests and sampling times were more significant overall. An increment in DNA-SB at $T_{14}$ compared with $T_0$ was found to be significant even for the tests with sediments A and B, which was not evident from ENA analysis (Fig. 3.1.7). Nevertheless, only test B showed evidence of a significant increase in DNA-SB from$T_{14}$ to $T_{28}$ (Mann-Whitney *U* test, $p < 0.05$).

**Table 3.1.2.** Categorized concentrations of PAHs, PCBs and DDTs in the tested sediments A (reference), B and C.

| | | Concentration in sediment ($ng.g^{-1}$) | | |
|---|---|---|---|---|
| | **Substance** | **A** | **B** | **C** |
| **PAHs** | *3 - ring* | | | |
| | Acenaphthylene<br>Acenaphthene<br>Fluorene<br>Phenanthrene<br>Anthracene | 11.95 ± 2.03 | 81.32 ± 13.82 | 83.60 ± 14.21 |
| | *4 - ring* | | | |
| | Fluoranthene<br>Pyrene<br>Benz[a]anthracene<br>Chrysene | 39.43 ± 6.70 | 395.46 ± 67.23 | 479.40 ± 81.50 |
| | *5 - ring* | | | |
| | Benzo[b]fluoranthrene<br>Benzo[k]fluoranthrene<br>Benzo[e]pyrene<br>Benzo[a]pyrene<br>Dibenzo[a,h]anthracene<br>Perylene | 24.35 ± 4.14 | 227.06 ± 38.60 | 266.06 ± 45.03 |
| | *6 - ring* | | | |
| | Benzo[g,h,i]perylene<br>Indeno[1,2,3-cd]pyrene | 5.99 ± 1.02 | 91.55 ± 15.56 | 62.26 ± 10.58 |
| **PCBs** | *Trichlorinated* | | | |
| | CB 18<br>CB 26<br>CB 31 | 0.73 ± 0.12 | 0.33 ± | 0.17 ± |
| | *Tetrachlorinated* | | | |
| | CB 44<br>CB 49<br>CB 52 | 0.13 ± 0.02 | 0.58 ± 0.10 | 0.81 ± 0.14 |
| | *Pentachlorinated* | | | |
| | CB 101<br>CB 105<br>CB 118 | 0.08 ± 0.01 | 1.49 ± 0.25 | 6.76 ± 1.15 |
| | *Hexachlorinated* | | | |
| | CB 128<br>CB 138<br>CB 149<br>CB 151<br>CB 153 | 0.43 ± 0.07 | 1.57 ± 0.27 | 7.22 ± 1.23 |
| | *Heptachlorinated* | | | |
| | CB 170<br>CB 180<br>CB 194 | 0.30 ± 0.05 | 0.95 ± 0.16 | 0.38 ± 0.06 |
| **DDTs** | DDE | 0.05 ± 0.01 | 0.27 ± 0.05 | 0.65 ± 0.11 |
| | DDD | 0.10 ± 0.02 | 0.28 ± 0.05 | 0.60 ± 0.10 |
| | DDT | 0.70 ± 0.12 | 4.39 ± 0.74 | 1.18 ± 0.20 |

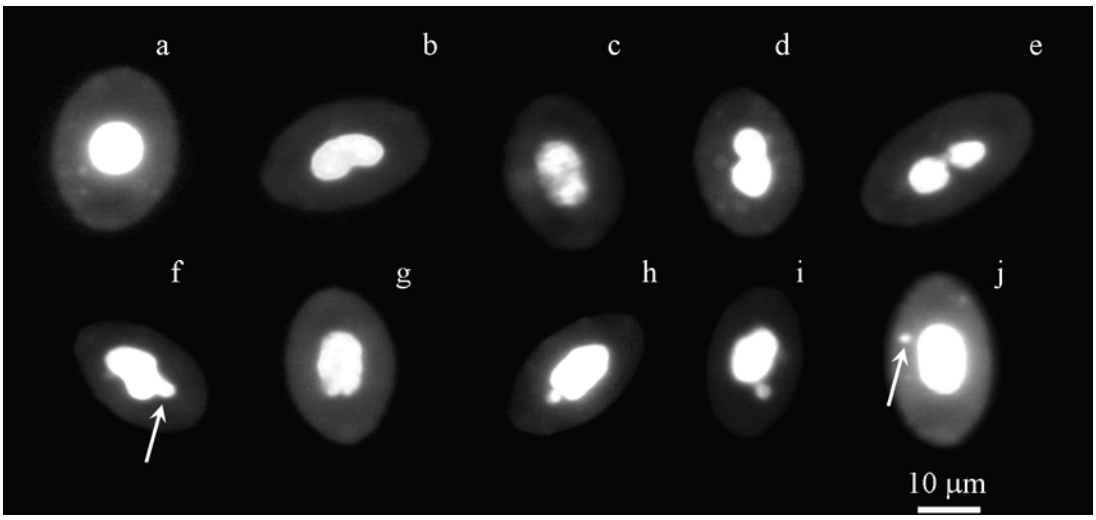


**Fig. 3.1.4.** Common ENA observed in mature erythrocytes of exposed *S. senegalensis*. (a) Normal mature erythrocyte, (b) lobed nucleus, (c-e) stages of fragmenting nuclei to form a binucleated cell, (f) lobed nucleus with nuclear bud (arrow), (g-j) different stages of micronucleus formation from a nuclear bud to a fully individualized micronucleus (arrow).

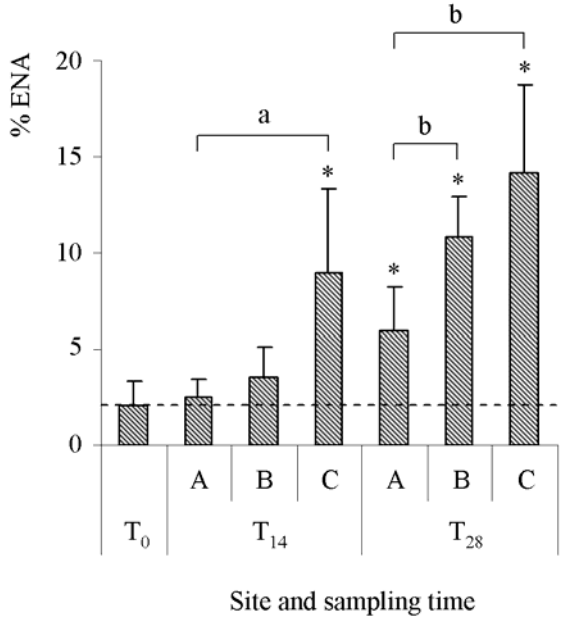


**Fig. 3.1.5.** Average percentage of cells showing ENA. Error bars represent 95% confidence intervals. (*) indicates differences ($p < 0.01$, Mann-Whitney *U* test) from $T_0$ (dashed line). a and b indicate differences between tests (Mann-Whitney *U* test): $p < 0.01$ and $p < 0.05$, respectively.

Spearman rank-order correlations confirmed sediment PAHs and PCBs of freshly collected sediments to be the variables that mostly influence the increase of genotoxic effects, at both $T_{14}$ and $T_{28}$. At $T_{28}$, however, Cu, As, Cd and Pb were also found positively correlated with both ENA and DNA-SB (Table 3.1.3). No significant correlations (Spearman *R*, $p > 0.05$) were found between body size (total wet weight, $ww_t$, and $L_S$) and both ENA and DNA-SB.

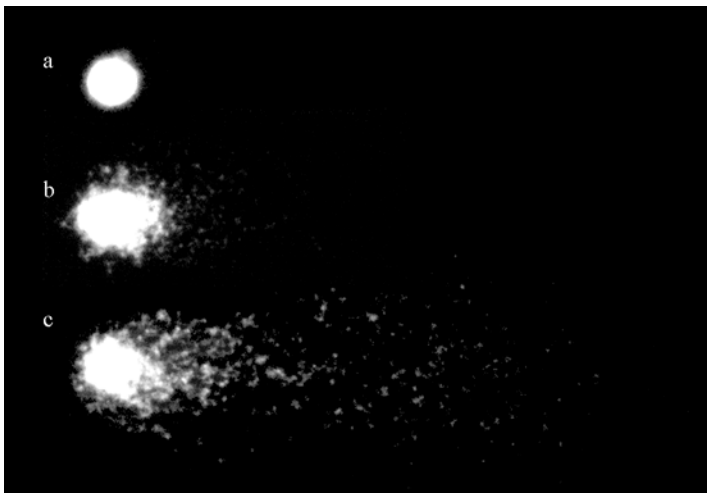


**Fig. 3.1.6.** Comet examples from tested fish: ≈ 0% (a), ≈ 20% (b) and ≈ 55% (c) DNA-SB.

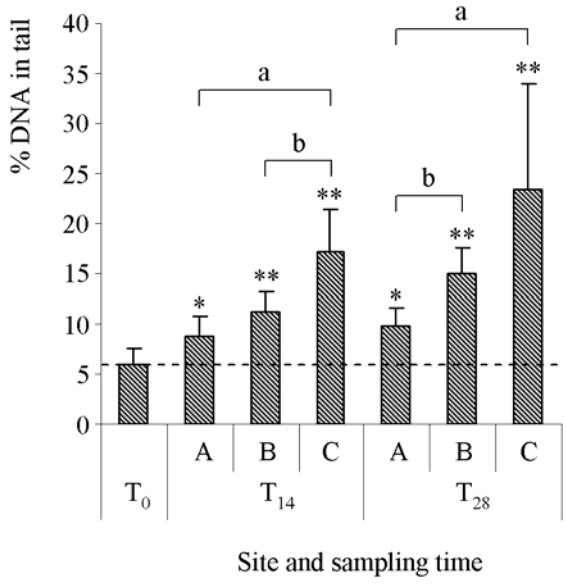


**Fig. 3.1.7.** Average percentage of DNA in tail. Error bars represent 95% confidence intervals. (*) and (**) indicate differences from $T_0$ (dashed line): $p < 0.05$ and $p < 0.01$, respectively. a and b indicate differences between tests (Mann-Whitney *U* test): $p < 0.01$ and $p < 0.05$ respectively.

## 4. Discussion and conclusions

The sediment with higher concentrations of organic contaminants (sediment C) was responsible for faster and stronger genotoxic effects and caused the highest overall mortality, whereas the sediment most heavily contaminated with metals (sediment B) was found to induce genotoxicity more significantly at a later stage and to cause less lethality. Differences between test sediments appear to be linked to the nature of the contamination (organic/metallic). The difference between genotoxic effects of both classes of contaminants may be explained by two factors: (i) the stronger

genotoxicity of organic toxicants, especially PAHs and PCBs and (ii) the known inhibitory action of metals towards the cytochrome P450 (CYP) isoenzymes involved in the catabolism of organic compounds.

**Table 3.1.3.** Spearman rank-order correlations for sediment contaminants × genotoxicity biomarkers (n.s., non--significant).

| | | % ENA | | % DNA SB | |
|---|---|---|---|---|---|
| | | Spearman *R* | *p* | Spearman *R* | *p* |
| $T_{14}$ | Cr | - | n.s. | - | n.s. |
| | Ni | - | n.s. | - | n.s. |
| | Cu | - | n.s. | - | n.s. |
| | Zn | - | n.s. | - | n.s. |
| | As | - | n.s. | - | n.s. |
| | Cd | - | n.s. | - | n.s. |
| | Pb | - | n.s. | - | n.s. |
| | tPAH | 0.37 | < 0.05 | 0.51 | < 0.01 |
| | tPCB | 0.37 | < 0.05 | 0.51 | < 0.01 |
| | tDDT | - | n.s. | - | n.s. |
| $T_{28}$ | Cr | - | n.s. | - | n.s. |
| | Ni | - | n.s. | - | n.s. |
| | Cu | 0.44 | < 0.01 | 0.45 | < 0.01 |
| | Zn | - | n.s. | - | n.s. |
| | As | 0.44 | < 0.01 | 0.45 | < 0.01 |
| | Cd | 0.44 | < 0.01 | 0.45 | < 0.01 |
| | Pb | 0.44 | < 0.01 | 0.45 | < 0.01 |
| | tPAH | 0.54 | < 0.001 | 0.59 | < 0.001 |
| | tPCB | 0.54 | < 0.001 | 0.59 | < 0.001 |
| | tDDT | 0.44 | < 0.01 | 0.45 | < 0.01 |

PAHs like benzo[a]pyrene (B[a[P), one of the best represented types of PAH in sediments B and, especially, C, are known sediment genotoxicants that have been widely employed in mutagenicity and carcinogenicity studies *in vitro*, in laboratory assays (using spiked or actual field-collected water and sediments) and in field-collected or laboratory-tested animals, animal cell lines and microorganisms (Chen and White, 2004). Studies with benthic fish, especially flatfish, have also become common for research on sediment PAHs (Myers et al., 2003). These compounds are potent DNA-SB inducers at low doses: PAHs in water, for instance, have been found to induce DNA damage in fish liver and erythrocytes even at concentrations as low as 0.3-0.5 ppb (Aas et al., 2000; Neuparth et al., 2006). PAHs are known to have severe mutagenic and carcinogenic effects (Chen and White, 2004; Myers et al., 2003) by binding to DNA (adduct formation) promoting DNA instability and potentially giving rise to strand breakage, but the xenobiotic-DNA adduct formation is dependent on PAH activation by cytochrome P450. The liver is the most important organ involved in contaminant metabolism and for this reason the P450 activity in the liver and genotoxic effects in hepatocytes are commonly surveyed in relation with contamination with PAH and other pollutants. ROS-inducing metabolites and activated PAHs may enter the blood stream and affect blood cells and haematopoietic organs, e.g., by inducing DNA strand-breakage directly and by formation of xenobiotic-DNA adducts, respectively. For this reason research on peripheral blood and bone marrow is highly relevant. Furthermore, it is likely that blood is rapidly affected by the direct action of contaminants, as it is the main conveyor of xenobiotics.

The nature of the defence mechanisms against PAH contamination may also contribute to explain the observed results regarding DNA-SB. Even though benthic fish species display a fast PAH catabolism upon contamination (Varanasi and Gmur, 1981), PAH-DNA adducts in fish liver appear to be repaired slowly (Aas et al., 2000; Ploch et al., 1998), which may have contributed to a cumulative effect in DNA damage along the assay's timescale, especially in tests of sediment C, where the most significant increase in genotoxicity (even at $T_{14}$) was observed, which may have further compromised the efficiency of DNA repair. On the other hand, genotoxicity caused by PAHs may occur as an effect of overexpression of cytochrome P4501A (CYP1A)-related isoenzymes by (i) activation of the PAHs, e.g., through formation of PAH quinones (Flowers-Geary et al., 1996), which allows them to bind directly to DNA (forming PAH-DNA adducts) and (ii) by formation - during PAH catabolism - of ROS, which are responsible for direct DNA base oxidation (Risom et al., 2005; Hansen et al., 2007). The combination of these factors may have contributed to a faster induction of genotoxicity, and causing the highest mortality, in the assay with sediment C. It is noteworthy, though, that metals in sediment B, which also contains high levels of PAH when compared with sediments from site A, may have inhibited P450 activity instead of inducing it, therefore reducing genotoxicity of PAHs by restraining the formation of the highly genotoxic activated PAH metabolites. This is in accordance with previous findings regarding CYP induction by exposure to PAH and metals in the liver of fish and other vertebrates, and showing that metals with high inhibitory capability are Cd >As > Pb and also Zn and Cu (Brüschweiler et al., 1996; Vahkaria et al., 2001; Lewis et al., 2006). Since our

findings strongly suggest a relationship between overall mortality and genotoxicity, it is likely that a reduction in PAH and PAH metabolites may significantly decrease lethality.

Information is scarce regarding the mechanisms of chromosomal breakage (leading to the formation of micronuclei and other nuclear abnormalities), but PAHs in water have since long been described as strong inducers of micronuclei in fish erythrocytes (Hose et al., 1984), as well as PCBs and DDTs (Hose et al., 1987). Exposure to complex mixtures of organic contaminants such as petroleum effluents in water has also been found responsible for ENA induction in fish (Ergene et al., 2007). Due to the chromosomal (clastogenic) nature of ENA (as opposed to the nucleotide-related nature of DNA-SB), research has essentially focused on the effects of organic compounds such as colchicines (see, e.g., Rodriguez-Cea et al., 2003), known for its effect on cell division by affecting the spindle apparatus. Our results show, nevertheless, that PAHs and PCBs are linked to ENA and, therefore, that these substances are capable of inducing chromosome-level genotoxicity in parallel to DNA-SB, and consequently are similarly related to the observed lethality. No effect was detected for DDTs, most likely due to the low concentrations found in all tested sediments.

Some metals (like Cd) and metalloids (like As) are also known as strongly genotoxic substances but, along with Pb and Cu, they were observed to have a slower, but almost as strong effect on ENA and DNA-SB at $T_{28}$. This was even the case for sediment B, which presents a lower level of contamination by organic compounds than sediment C. This delayed but strong effect may be related to the difference between the mechanisms of metal- and organic-induced genotoxicity and a slower release of metals from sediments during the assay. Metal genotoxicity regarding DNA-SB seems to be more directly linked to the formation of ROS, e.g., induced by metallic metabolites like some methylated forms of As (Soto-Reyes et al., 2005).

The effects of metallic ions on the formation of ENA are well known and long established for toxicology tests *in vitro* and *in vivo*. Cd, As, Pb and Cu are strong ENA inducers (Ayllon and García-Vázquez, 2000; Çavas et al., 2005; Gebel, 2000; Palus et al., 2003). Nevertheless, the genotoxicity mechanisms of ENA formation as a result of metal exposure are not yet fully understood and may vary with different metals. Some evidence points to a correlation of ROS and antioxidant enzymes with micronuclei upon As exposure (see, e.g., Wang et al., 1997), but it is generally accepted that induction of micronuclei is essentially dependent on the role of xenobiotics on DNA replication and cell division (Nikinmaa, 1992), which may be a reason why the comet assay appeared to be more sensitive at $T_{14}$, since direct DNA-SB may be more rapidly induced. The interactions between organic and metallic contaminants on ENA are unclear, but recent findings suggest that B[a]P-mediated induction of micronuclei in mouse bone-marrow may be enhanced by simultaneous exposure to As, but not Cd (Lewińska et al., 2007). Our results, nevertheless, suggest a possible inhibitory action of metals on organic contaminant-induced ENA, as observed for DNA-SB.

Sediments B and C have higher levels of TOM and FF, while metals are known to have a high affinity for sediment organic matter and FF (Caccia et al., 2003; Mota et al., 2005). The bioavailability of some metals may have increased progressively during the assays due to alterations of the redox

status of the sediments and extensive re-suspension of sediments caused by the scavenging activities of the fish. It is noteworthy that highly toxic metals like Cd may have a prolonged availability in the water column after sediment re-suspension (Caetano et al., 2003). Sediments from site B and, even more so, site C had more reducing conditions (lower Eh) that, combined with re-suspension, should have favoured the release of both organic and metallic contaminants (Eggleton and Thomas, 2004) and thus contributed to the much stronger genotoxic effects and lethality of sediments B and C in comparison with those of sediment A. Genotoxicity may have in fact been enhanced in all three tests (hence a significant DNA-SB increase in fish exposed to sediment A from $T_0$ to $T_{14}$) just by a combination of low Eh and re-suspension during the assays, which may have favoured the bioavailability of the contaminants.

The metal concentrations in the three sediments are in accordance with previous contaminant profiles obtained for the study area (Caeiro et al., 2005), but concentrations measured in the present study were overall lower. Nonetheless, resuspension and alterations of the sediment parameters during the assays render it difficult to extrapolate risk assessment from sediment contaminants to the natural environment, due to alterations in bio-availability and the presence of other contaminants, an issue that has been established as being of special relevance with respect to the combined presence of metallic and organic contaminants (Woodhead et al., 1999). A comparison with previous data from sediments collected from approximately the same sites revealed that tPAHs decreased tenfold for all sites, but tPCBs increased tenfold. As in the present study, this comparison identified the PAHs as the type of toxicant most likely implicated in a significant induction in DNA-SB in the test organisms (Neuparth et al., 2005). These findings confirm the high genotoxicity of PAHs and their relevance among the organic contaminants in the different sediments surveyed, both with respect to concentration and toxicity. Other studies have found a probable relation between sediment PAHs (i.e., the most representative contaminants) and genotoxicity in benthic fish (Kilemade et al., 2004), but research on natural sediments that integrates both organic and metallic contaminants and their interactions on genotoxicity is essentially lacking. This is yet another factor that renders it difficult to assess the actual genotoxic potential of sediment mixtures of contaminants in fish and to define toxicity thresholds.

In general, the results from both sediment characterization and biomarker analyses reflect the expected contamination patterns for the three stations. Concentration of contaminants in sediments from sites B and C are likely to be related with proximity of pollutant “hotspots”, namely from urban and port facilities (metals in B) and industry, especially chemical plants and runoffs from agriculture grounds upstream likely to be contaminated with fertilizers and pesticides (organic contaminants in C). Furthermore, higher levels of FF and TOM in sediments B and C may have allowed higher accumulation of contaminants, later released during the assays.

The use of ENA (analysed with fluorescence techniques) and DNA-SB (measured with the comet assay) as genotoxicity biomarkers proved successful in the context of a practical biomonitoring procedure for contaminated estuarine sediments and also allowed assessment of the relative genotoxic effects of metals and organic contaminants in sediments. The comet assay appeared to be a more

sensitive method than ENA analysis, since it reflected better the contamination pattern and the observed lethality from the test sediments, contradicting some of the results from others, who have questioned the sensitivity and applicability of the comet assay as a biomonitoring tool for aquatic contamination (Wirzinger et al., 2007; Bombail et al., 2001). It is concluded that genotoxicity assessment in peripheral blood cells of a benthic vertebrate such as *S. senegalensis* is a valuable tool for biomonitoring estuarine sediments contaminated with mixed classes of substances.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors would like to thank T. Neuparth (CIIMAR), APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and RNES (Reserva Natural do Estuário do Sado) for the logistic support and Pedro Pousão (Estação Piloto de Piscicultura, INRB/IPIMAR-Cripsul).

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## 3.2. Biochemical endpoints on juvenile *Solea senegalensis* exposed to estuarine sediments: the effect of contaminant mixtures on metallothionein and CYP1A induction[†]


### Abstract

Juvenile *Solea senegalensis* were exposed to fresh sediments from three stations of the Sado Estuary (Portugal) in 28-day laboratory assays. Sediments revealed distinct levels of total organic matter, fine fraction, redox potential, trace elements (arsenic, cadmium, chromium, copper, nickel, lead and zinc) and organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls and a pesticide: dichloro diphenyl trichloroethane). Organisms were surveyed for contaminant bioaccumulation and induction of two hepatic biochemical biomarkers: metallothionein (MT) and cytochrome P450 (CYP1A), as potential indicators of exposure to metallic and organic contaminants, respectively. Using an integrative approach it was established that, although bioaccumulation is in general accordance with sediment contamination, lethality and biomarker responses are not linearly dependent of the cumulative concentrations of sediment contaminants but rather of their bioavailability and interaction effects in organisms. It is concluded that metals and organic contaminants modulate both MT and CYP1A induction and it is suggested that reactive oxygen species may be the link between responses and effects of toxicity.



### Keywords

Senegalese sole; Contaminated sediments; Bioaccumulation; Metallothionein; CYP1A; Sado Estuary


### 1. Introduction

Ecological risk assessment of aquatic sediments has many constraints related to the difficulty in determining cause-effect relationships when toxicity results from mixtures of contaminants and the sediment characteristics that determine their bioavailability (Chapman 1990). Although laboratory and *in situ* tests using a wide range of aquatic organisms have proven its usefulness to assess the ecological risk of dredged materials and natural-state sediments, much research is still in need to fully understand the effects and implications of the toxicity of such a complex media. Also, even though benthic fish

[†] Costa et al. (2009). *Ecotoxicology* **18**, 988-1000 (doi:10.1007/s10646-009-0373-7).

such as the Senegalese sole, *Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae), have been successfully employed in bioassays with contaminated sediments (Jiménez-Tenorio et al., 2007; Costa et al., 2008a), little research has been able to relate toxicity effects and responses to specific classes of mixed sediment contaminants or their interactions.

Metallothionein or metallothionein-like proteins (MTs) are small cytosolic heat-resistant proteins (≈ 6-7 kDa) formed by two globular subunits, each comprising a high content (about 20) of free thiolic (-SH) groups from cysteine residues that are able to sequester metallic ions. The structure of these ubiquitous, highly conserved, proteins is linked to their role in the homeostasis of essential metals such as zinc (Zn) and copper (Cu) and detoxification of toxic elements such as cadmium (Cd) and mercury (Hg). For these reasons, gene overexpression (induction) of MTs has long been proposed as a biomarker of metal exposure, since metallic ions are capable of activating a metal response transcription factor (MTF) which will promote MT gene transcription by binding to specific DNA sites. MTs have several isoforms, apparently induced by different metals, the best known of which (MT1 and MT2) are greatly induced by Cd and Zn (reviewed by Viarengo et al., 1999; Romero-Isart and Vašak 2002).

Cytochrome P4501A (CYP1A) consists of a membrane enzymatic system of monooxygenases involved in the catabolism of organic toxic xenobiotics such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). These toxicants have the ability to induce CYP1A gene transcription by binding to a specific receptor, the aryl hydrocarbon receptor (AhR). Determination of CYP1A protein content or activity has been widely used as biomarker of exposure to organic contaminants in a broad range of organisms. An important characteristic of CYP1A catalytic activity is the formation of highly toxic (but more soluble, thus more easily excretable) intermediate products (many of which are strongly genotoxic) and reactive oxygen species (ROS); substances that are responsible for a diffuse and non-specific toxicity (see Jönsson et al., 2009 for a recent review).

The Sado Estuary is a large coastal area subjected to various anthropogenic pressures, from urbanistic to industrial (comprising one of the highest heavy-industry concentrations in Portugal) and also agriculture (at upstream grounds), aquaculture, tourism and fisheries. Part of the estuary is also classified as a natural reserve. The obvious conflict between anthropogenic exploitation and the need to preserve environmental quality ultimately determine the importance of implementing ecological risk assessment strategies.

The main goals of the present work consist of: (1) to assess chronic sediment toxicity to juvenile *S. senegalensis* through analysis of bioaccumulation and MT and CYP1A induction in the liver, typically considered as toxicity biomarkers for metals and organic compounds, respectively; (2) to integrate sediment parameters and contaminants with the effects and responses in fish to assess toxicant bioavailability and (3) to evaluate the effects and responses of sediment-based contaminant mixtures to the organisms.

## 2. Materials and methods

### *2.1. Experimental design*

The three sites of the Sado Estuary (Fig. 3.2.1) were chosen according to their distinct characteristics: site A (the reference location) is the closest to the Natural Reserve and the farthest from pollution sources and has the smallest water residence time, therefore being the least contaminated, whereas sites B and C, located off Setúbal's harbour and industrial belt, respectively, are potentially the most contaminated. Sediments were collected with a Petite Ponar grab on November 2006 and were analyzed immediately for their redox potential (Eh), homogenized and subdivided into samples to be frozen for posterior analyses or to be used fresh in the assays. For simplification purposes, exposure to the three sediments is throughout referred to as tests A, B and C.

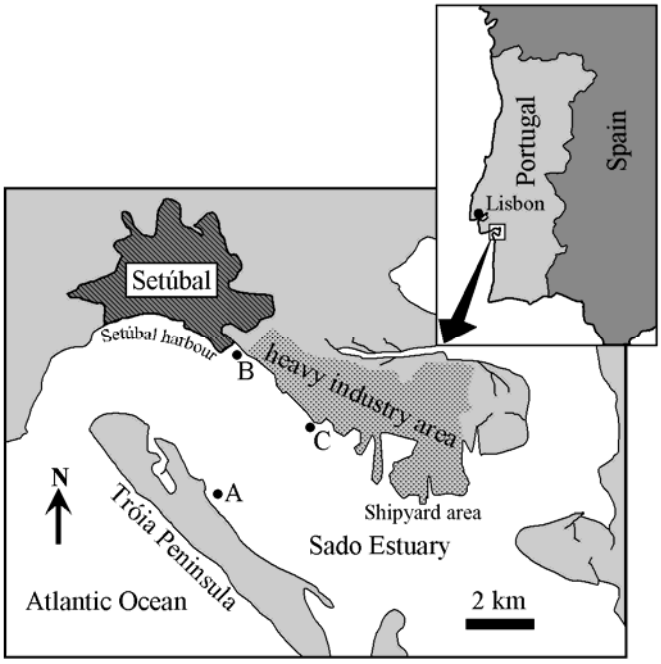



**Fig. 3.2.1.** Map of the Sado Estuary showing the three sediment collection sites, A, B and C (•).

For each test, 2 L of sediment were placed in 15 L polyvinyl tanks (providing ≈ 525 $cm^2$ of sediment surface) with 12 L of clean seawater and let settle for 48 h prior to the beginning of the assays. Assays were dynamic, with constant aeration, and performed in duplicate. Water flow was set to prevent hydrodynamic-driven sediment resuspension. A quarter of the total water volume was changed weekly in order to mimic and keep constant the animals' rearing conditions with minimal removal of suspended matter or waterborne contaminants. Measured parameters throughout the duration of the assays were: pH 7.9 ± 0.2, salinity = 33 ± 1, temperature = 18 ± 1 ºC, dissolved $O_2$ ($DO_2$) ranged between 40 and 45% and total ammonia was restrained within 2 - 4 $mg.L^{-1}$. Photoperiod was set at 12:12 h light:dark. The water parameters were monitored weekly and simultaneously a

water sample was taken for vacuum filtration with a GFC filter to determine the amount of suspended particulate matter.

Twenty-four juvenile hatchery-brood and laboratory reared *S. senegalensis* [69 ± 6 mm standard length ($L_s$)], all from the same cohort, were randomly distributed by each tank. Animals were fed daily with M2 grade commercial fish pellets (AQUASOJA) for the duration of the assay. Sampling times were scheduled for days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$). At sampling times $T_{14}$ and $T_{28}$ twelve animals per exposure treatment (six per replica) were sacrificed, measured for $L_s$ and total wet weight ($ww_t$) and the liver was excised for biomarkers and metal bioaccumulation analyses. Remaining animals (up to six per replica, depending on mortality) were analysed for organic contaminant bioaccumulation. $T_0$ individuals consisted of 12 fish collected directly from the rearing tanks.

*2.2. Sediment characterization*

Sediments were characterized for total organic matter (TOM) by complete ignition at 500 ± 50 ºC. Fine fraction (FF, particle size < 63 μm) was determined by hydraulic sieving, as described by Caeiro et al., (2005). Both results are expressed as % relatively to sediment dry weight (dw).

Sediment metallic elements were determined from dry samples mineralized in acid (6 mL HF 40% v/v to which was added 1 mL of the mixture 36% HCl plus 60% $HNO_3$ 3:1 v/v) in closed Teflon vials (Caetano et al., 2007). Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) were quantified by inductively coupled plasma mass spectrometry (ICP-MS). MESS-2 (NRC, Canada), PACS-2 (NRC, Canada) and MAG-1 (USGS, USA) reference sediments were analyzed to validate the procedure and the obtained metal concentrations were found within the certified range.

Sediment PAHs were analyzed from dry samples spiked with surrogate standards (Supelco), Soxhlet-extracted with an acetone + hexane (1:1 v/v) mixture and quantified by gas chromatography--mass spectrometry (GC-MS) as described by Martins et al. (2008). Seventeen 3- to 6- -ring PAHs were quantified, with total PAHs (tPAH) meaning the sum of all individual PAHs. PCBs (18 congeners) and DDTs (*pp*'DDT plus the metabolites *pp*'DDD and *pp*'DDE) were quantified from dried sediment samples Soxhlet-extracted with n-hexane, fractioned in a chromatographic column and quantified by GC with an electron capture detector (Ferreira et al., 2003). tPCB and tDDT represent the sum of the concentration of all analyzed PCB congeners and DDTs, respectively. Validation of the procedure was obtained by analysis of the SRM 1941b reference sediment (NIST, USA) and the concentrations of surveyed organic compounds were found within the certified range.

*2.3. Bioaccumulation*

Metal bioaccumulation was determined from vacuum-dried (24 h) liver samples from each individual, digested in closed Teflon vials with $HNO_3$ and $H_2O_2$, according to Clesceri et al. (1999).

The concentrations of a metalloid (As) and six metals (Cd, Cr, Cu, Ni, Pb and Zn) were determined by ICP-MS. DORM2 and DOLT3 reference materials (NRC, Canada) were used to validate analysis and the obtained values were found within the certified range. In order to ensure enough biomass to survey PAHs, PCBs and DDTs (a minimum of ≈ 5 g per Soxhlet extraction was required), organic contaminants were quantified in wet muscle samples pooled from up to twelve fish. Quantification was performed similarly to the procedure described in the previous section, adapted to biological tissue by Martins et al. (2008). Validation was obtained by analysis of reference mussel tissue SRM 2977 (NIST, USA) and values were found to be within the certified range.

*2.4. Biomarker analyses*

Metallothionein induction was assessed by quantification of thiols in individual heat-treated liver cytosols (80 ºC, 10 min) extracted in Tris-HCl 0.02 M buffer (pH 8.6), by differential pulse polarography with a static mercury drop electrode (DPP-SMDE), as described by Costa et al. (2008b). In absence of an available commercial fish MT, rabbit MT (isoforms 1 and 2) from Sigma was used for standard addition. The presence of a MT-compatible protein in liver cytosols was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), through comparison of cytosolic protein migration distances with rabbit MT and the Pre-Stained Broad-Range protein ladder (Bio-Rad) using the software Quantity One (also from Bio-Rad). Due to difficulties in SDS-PAGE visualization of MTs, a specific method described by Costa and Costa (2008) was employed, using a 19% acrylamide/bis-acrylamide running gel, and gels were stained by the blue-silver method with Coomassie brilliant blue G250 (Candiano et al., 2004).

CYP1A induction was determined from pooled liver samples (of 12 individuals) for each test and sampling time, in order to have sufficient tissue mass for analysis (50-100 mg). Quantification was assessed by enzyme-linked immunosorbent assay (ELISA) in the microsomal fraction of liver homogenates, according to Nilsen et al. (1998) using a polyclonal antibody (CP226) against fish CYP1A (Biosense). CYP1A extraction and quantification was performed in triplicate to determine the error of procedure. In absence of a commercial fish CYP1A standard, results are given in % of CYP1A induction over $T_0$.

*2.5. Statistical analyses*

Correlation-based principal component analysis (PCA) was employed to discriminate the most relevant variables explaining the variance between tested fish. A multivariate approach to integrate all biological data, namely fish size, bioaccumulation and biomarkers was performed through generalized linear models (GLM) in order to create a MT induction model based on biological parameters. The model was fitted through a Gamma regression (bioaccumulation + CYP1A induction + $ww_t \times L_s$ interaction) with an identity link. Statistical inference on explanatory variables (model components)

was obtained by ANOVA based on the reduction of deviance by sequential variable addition. Inference on coefficients was done by the Student's *t*-test. The model was validated by the criteria of randomness of Pearson (standardized) residuals × fitted values, normality of Pearson residuals and Cook's statistic *h* for assessment of individual case biasing on model estimates. Observed × predicted chi-square tests were used to assess differences between sampling times of sediment contaminants and organic compound bioaccumulation. Non-parametric statistics (Kruskall-Wallis *H*, Mann-Whitney *U* and Spearman's rank-order correlation *R*) were employed whenever the Levene's and Kolmogoroff--Smirfnoff's tests failed to demonstrate the homogeneity of variances or normality of data assumptions, respectively. GLM analysis was performed with the software R (Ihaka and Gentleman 1996); all other statistics were computed with Statistica (Statsoft), following McCullagh and Nelder (1989) and Sheskin (2000), respectively.

## 3. Results

The three tests yielded very distinct mortalities at the end of the assays: test C was responsible for the highest lethality (48%), followed by B (12%) and A (2%). Growth at the end of the experiment was not found significant when compared to $T_0$ and also between tests and replicates (Kruskall-Wallis *H*, $p < 0.05$). Average $L_s$ and $ww_t$ at the end of each sampling time were 70 ± 6 mm and 4.21 ± 1.53 g, respectively.

### *3.1. Sediment characterization*

Burying and scavenging activities of the animals were observed to cause a strong resuspension of the sediments that progressively decreased during the assays, most likely due to the periodical water changes and to the dynamic structure of the assays, leading to a decrease in suspended particulate matter (Fig. 3.2.2).

Sediments from the three sites revealed distinct characteristics and contamination patterns (Table 3.2.1). Sediment C was found to be the most anoxic and B with the highest TOM and FF percentages. As expected, sediment A is the overall least contaminated, while B is the most contaminated by metals. A comparison between the measured contaminant concentrations and available sediment quality guidelines (*SQG*s), namely the threshold effects level (*TEL*) and the probable effects level (*PEL*) confirmed sediment A to be the least contaminated, with sediments B and C being moderately contaminated, the first reaching *PEL* thresholds for Cu and Zn. Four- and five--ring PAHs were the best represented organic toxicants, with perylene in sediment C reaching much higher levels (more than twofold) than in B. The phenanthrene/anthracene and fluoranthene/pyrene ratios were > 1 and < 10, respectively, for all sediments, indicating the essentially pyrolytic (combustion-derived) origin of PAHs. PCBs were consistently more concentrated in sediment C when

compared to A and B. DDT (and its metabolites) was the least represented organic contaminant, presenting higher levels in sediment B.

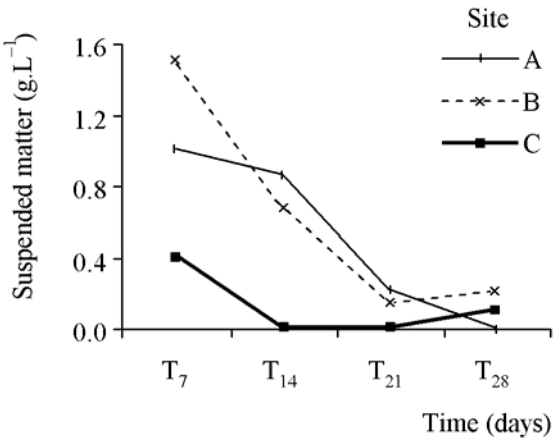



**Fig. 3.2.2.** Time-point measurements of total suspended matter (at $T_7$, $T_{14}$, $T_{21}$ and $T_{28}$). Points are connected to illustrate the evolution of suspended particles throughout the assays.

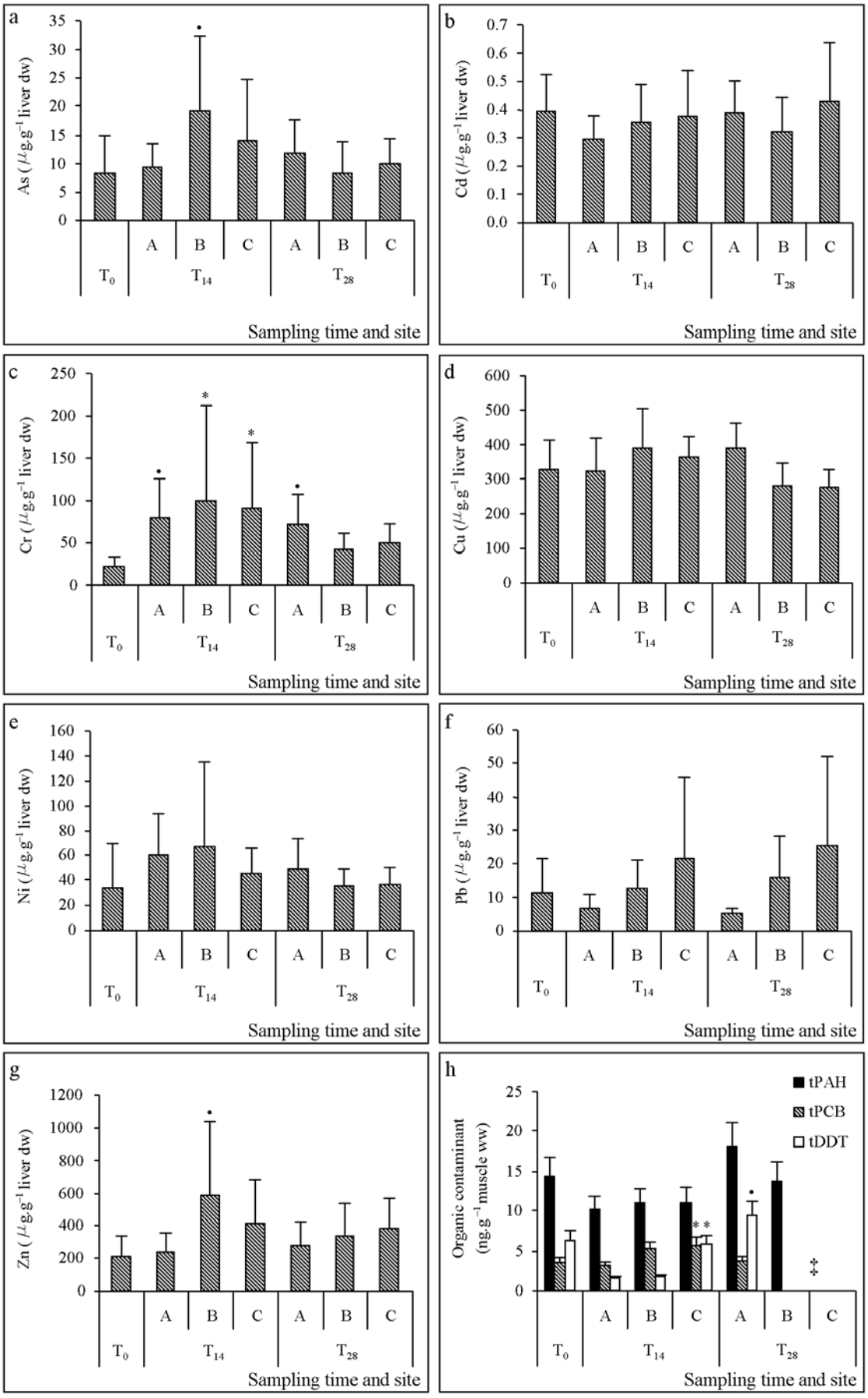
*3.2 Bioaccumulation*

Metal bioaccumulation in the liver was in general low for all tests but more evident at $T_{14}$, especially for test B (Fig. 3.2.3a-g). Bioaccumulation was most notorious for As, Cr and Zn. Lead accumulation was found to be the most variable and did not account for any statistically significant trends. No differences were found between replicates for all analysed elements (Mann-Whitney *U*, $p < 0.05$), so data from the two replicates were grouped for further statistics. Comparatively to $T_0$, differences in muscle organic toxicants (Fig. 3.2.3h) were more evident for test C, especially regarding PCBs and DDTs, at $T_{14}$, even though data is incomplete due to the mortality in B and C tests, which caused the lack of minimum muscle mass for analysis. From the analysis of individual organic contaminants (Table 3.2.2) it may be inferred that the differences between sampling times regarding test C are caused by an increase in accumulated *pp*'DDD and *pp*'DDE (approximately twofold from $T_0$ to $T_{14}$) and tri- to pentachlorinated PCBs and a decrease in *pp*'DDT to values below the detection limit. The opposite was observed in A-tested fish at $T_{28}$, where a more than twofold increase in muscle pp'*DD*T was observed. PAHs failed to depict any significant variation between sampling times for all tests but it was observed that fish accumulated lower molecular weight PAHs (3-ring) and not the 4- to 5-ring forms that are the most representative PAHs in the sediments, a result that is similar to studies with other marine organisms (Bihari et al., 2007). In general, bioaccumulation of both metals and organic compounds appears to reflect sediment contamination.

**Table 3.2.1** Characterization of tested sediments and comparison of contaminant levels to available *TEL* and *PEL SQG*s, given in mg.kg$^{-1}$ sediment dw for metals and µg.kg$^{-1}$ sediment dw for organic substances (from MacDonald et al., 1996).

| | | | | | Site | | |
|---|---|---|---|---|---|---|---|
| | | | | | **A** | **B** | **C** |
| | | FF (%) | | | 37.3 | 97.9 | 76.8 |
| | | TOM (F) | | | 3.2 | 11.8 | 7.7 |
| | | Corrected Eh (mV) | | | -233 | -290 | -316 |
| Contaminant class | | | | *TEL*[†] | *PEL*[‡] | | | |
| Metallic (mg.kg$^{-1}$ sediment dw) | | | As | *7.24* | *41.6* | 7.25 ± 0.15* | 27.43 ± 0.55* | 12.38 ± 0.25* |
| | | | Cd | *0.68* | *4.21* | 0.04 ± 0.00 | 0.22 ± 0.00 | 0.15 ± 0.00 |
| | | | Cr | *52.3* | *160* | 24.20 ± 0.48 | 76.33 ± 1.53* | 21.85 ± 0.44 |
| | | | Cu | *18.7* | *108* | 22.57 ± 0.45* | 167.32 ± 3.35** | 41.18 ± 0.82* |
| | | | Ni | *15.9* | *42.8* | 12.97 ± 0.26 | 33.67 ± 0.67* | 9.03 ± 0.18 |
| | | | Pb | *30.2* | *112* | 23.70 ± 0.47 | 66.49 ± 1.33* | 45.17 ± 0.90* |
| | | | Zn | *124* | *271* | 147.48 ± 2.95* | 312.23 ± 6.24** | 87.75 ± 1.76 |
| Organic (µg.kg$^{-1}$ sediment dw) | PAH | 3 - ring | Acenaphthylene | *5.87* | *128* | 0.24 ± 0.04 | 1.83 ± 0.31 | 1.95 ± 0.33 |
| | | | Acenaphthene | *6.71* | *88.9* | 1.41 ± 0.24 | 9.42 ± 1.60* | 4.19 ± 0.71 |
| | | | Fluorene | *21.2* | *144* | 1.32 ± 0.22 | 8.70 ± 1.48 | 8.03 ± 1.37 |
| | | | Phenanthrene | *86.7* | *544* | 7.96 ± 1.35 | 50.77 ± 8.63 | 54.09 ± 9.20 |
| | | | Anthracene | *46.9* | *245* | 1.03 ± 0.17 | 10.60 ± 1.80 | 15.34 ± 2.61 |
| | | 4 - ring | Fluoranthene | *74.8* | *693* | 18.05 ± 3.07 | 170.80 ± 29.04 | 184.30 ± 31.33* |
| | | | Pyrene | *1538* | *1398* | 14.66 ± 2.49 | 131.74 ± 22.40 | 171.39 ± 29.14 |
| | | | Benz[a]anthracene | *74.8* | *693* | 4.53 ± 0.77 | 64.60 ± 10.98 | 86.52 ± 14.71* |
| | | | Chrysene | *108* | *846* | 2.20 ± 0.37 | 28.31 ± 4.81 | 37.19 ± 6.32 |
| | | 5 - ring | Benzo[b]fluoranthrene | *NG* | *NG* | 6.77 ± 1.15 | 60.86 ± 10.35 | 70.25 ± 11.94 |
| | | | Benzo[k]fluoranthrene | *NG* | *NG* | 4.16 ± 0.71 | 32.21 ± 5.48 | 40.18 ± 6.83 |
| | | | Benzo[e]pyrene | *NG* | *NG* | 5.12 ± 0.87 | 56.73 ± 9.64 | 62.76 ± 10.67 |
| | | | Benzo[a]pyrene | *88.8* | *793* | 7.56 ± 1.28 | 69.81 ± 11.87 | 85.88 ± 14.60 |
| | | | Perylene | *NG* | *NG* | 4.69 ± 0.80 | 86.97 ± 14.79 | 209.16 ± 35.56 |
| | | | Dibenzo[a,h]anthracene | *6.22* | *135* | 0.74 ± 0.13 | 7.45 ± 1.27* | 6.99 ± 1.19* |
| | | 6 - ring | Indeno[1,2,3-cd]pyrene | *NG* | *NG* | 4.87 ± 0.83 | 52.44 ± 8.91 | 51.82 ± 8.81 |
| | | | Benzo[g,h,i]perylene | *NG* | *NG* | 1.12 ± 0.19 | 39.12 ± 6.65 | 10.44 ± 1.78 |
| | | | **tPAH** | *1 684* | *16 770* | **86.4 ± 14.7** | **882.4 ± 150.0** | **1,100.5 ± 187.1** |
| | PCB | Trichlorinated | CB-18 | *NG* | *NG* | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.09 ± 0.01 |
| | | | CB-26 | *NG* | *NG* | 0.05 ± 0.01 | 0.06 ± 0.01 | 0.09 ± 0.01 |
| | | | CB-31 | *NG* | *NG* | 0.64 ± 0.11 | 0.19 ± 0.03 | < d.l. |
| | | Tetrachlorinated | CB-44 | *NG* | *NG* | 0.05 ± 0.01 | 0.38 ± 0.06 | < d.l. |
| | | | CB-49 | *NG* | *NG* | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.36 ± 0.06 |
| | | | CB-52 | *NG* | *NG* | 0.05 ± 0.01 | 0.12 ± 0.02 | 0.45 ± 0.08 |
| | | Pentachlorinated | CB-101 | *NG* | *NG* | 0.04 ± 0.01 | 0.23 ± 0.04 | 1.18 ± 0.20 |
| | | | CB-105 | *NG* | *NG* | 0.03 ± 0.01 | 0.22 ± 0.04 | 0.66 ± 0.11 |
| | | | CB-118 | *NG* | *NG* | < d.l. | 1.04 ± 0.18 | 4.92 ± 0.84 |
| | | Hexachlorinated | CB-128 | *NG* | *NG* | 0.01 ± 0.00 | 0.08 ± 0.01 | < d.l. |
| | | | CB-138 | *NG* | *NG* | 0.12 ± 0.02 | 0.68 ± 0.12 | 2.68 ± 0.46 |
| | | | CB-149 | *NG* | *NG* | 0.11 ± 0.02 | < d.l. | < d.l. |
| | | | CB-151 | *NG* | *NG* | 0.05 ± 0.01 | 0.17 ± 0.03 | 1.15 ± 0.20 |
| | | | CB-153 | *NG* | *NG* | 0.14 ± 0.02 | 0.64 ± 0.11 | 3.39 ± 0.58 |
| | | Heptachlorinated | CB-170 | *NG* | *NG* | 0.07 ± 0.01 | 0.27 ± 0.05 | < d.l. |
| | | | CB-180 | *NG* | *NG* | 0.21 ± 0.04 | 0.61 ± 0.10 | < d.l. |
| | | | CB-187 | *NG* | *NG* | 0.20 ± 0.03 | 0.72 ± 0.12 | < d.l. |
| | | | CB-194 | *NG* | *NG* | 0.03 ± 0.00 | 0.07 ± 0.01 | 0.38 ± 0.06 |
| | | | **tPCB** | *21.6* | *189* | **1.87 ± 0.32** | **5.64 ± 0.96** | **15.34 ± 2.61** |
| | DDT | | *pp*'DDE | *1.22* | *7.81* | 0.05 ± 0.01 | 0.27 ± 0.05 | 0.65 ± 0.11 |
| | | | *pp*'DDD | *2.07* | *374* | 0.10 ± 0.02 | 0.28 ± 0.05 | 0.60 ± 0.10 |
| | | | *pp*'DDT | *1.19* | *4.77* | 0.70 ± 0.12 | 4.39 ± 0.75* | 1.18 ± 0.20 |
| | | | **tDDT** | *3.89* | *51.7* | **0.85 ± 0.14** | **4.94 ± 0.84*** | **2.43 ± 0.41** |

[†]Threshold Effects Level - concentration below which contamination effects rarely occur; [‡]Probable Effects Level - concentration above which contamination effects frequently occur; [*], contaminant above *TEL*; [**], contaminant above *PEL*; [-], contaminant below sediment quality guideline (*SQG*); [*NG*], no guideline available; [< d.l.], below detection limit; Ranges indicate standard error.


a
As (μg.g⁻¹ liver dw)
b
Cd (μg.g⁻¹ liver dw)
c
Cr (μg.g⁻¹ liver dw)
d
Cu (μg.g⁻¹ liver dw)
e
Ni (μg.g⁻¹ liver dw)
f
Pb (μg.g⁻¹ liver dw)
g
Zn (μg.g⁻¹ liver dw)
h
Organic contaminant (ng.g⁻¹ muscle ww)
tPAH
tPCB
tDDT
T0
T14
T28
A
B
C
Sampling time and site

◀**Fig. 3.2.3.** Bioaccumulation results. * and • represent significant differences from $T_0$ individuals, $p < 0.05$ and $p < 0.10$, respectively. a-g) average concentration of metals in liver (Mann-Whitney *U*, error bars represent 95% confidence intervals, *n* = twelve fish per treatment and sampling time). h) concentration of major organic contaminant classes in pooled muscle samples (Chi-square Expected × Observed test between congeners, error bars indicate the standard error). ‡ indicates missing data due to mortality.

### *3.3. Biomarkers*

Metallothionein induction relatively to $T_0$ individuals was only observed at $T_{28}$ in tests B and, especially, C, despite that the sediment B presented higher levels of metal contamination (Fig. 3.2.4a). No differences were found between replicates (Mann-Whitney *U*, $p < 0.05$), so data from replicates were grouped for subsequent statistical analyses. Presence of a MT-compatible band in heat-treated cytosols was confirmed by SDS-PAGE (Fig. 3.2.4b). Estimated MW of *S. senegalensis* MT was 12 ± 1 kDa, which is consistent with vertebrate dimeric MTs. Estimated MW of rabbit standard MTs was 13 ± 1 kDa for the dimeric form and 5 ± 1 kDa for the monomeric form. Regarding CYP1A induction, however, tests with sediments A and B were responsible for the highest induction at $T_{14}$ (relatively to $T_0$), whereas at $T_{28}$ CYP1A levels of A- and B-tested individuals reverted to a $T_0$-like condition and C--tested animals showed a relatively small increment in CYP1A protein content (Fig. 3.2.4c).

### *3.4. Statistical integration of data*

Principal component analysis derived two main factors that combined explain 52.6% of all variance between tested animals. Analysis comprises all measured biological variables: bioaccumulation, biomarkers, $L_s$ and $ww_t$ (Fig. 3.2.5). The main contributors for factor 1, which explains the highest portion of within-individuals variance, were essentially accumulated metals, especially As, Cr, Ni and Zn. For factor 2 the main contributors were accumulated PAHs and DDTs plus CYP1A induction, although with a very distinct opposite trend between accumulated organic compounds and cytochrome induction. Analysis did not reveal MT induction as a significant contribution for either factor.

The liver MT induction model (validation shown in Fig. 3.2.6) retrieved liver Cr and Zn to be the most significant ($p < 0.05$) metals influencing MT induction but with opposite effects, having positive and negative coefficients (linear predictors), respectively. A similar pattern was detected regarding accumulated tPAHs (although less significantly) and tDDTs in muscle (Table 3.2.3). The effect of an interaction between $ww_t$ and $L_s$ ($ww_t$:$L_s$).was also found to be close to the level of significance but $L_s$ as an isolated variable did not significantly reduce model deviance whereas $ww_t$ only marginally did so.

**Table 3.2.2.** Bioaccumulation of individual organic contaminants in muscle of individuals exposed to sediments from sites A, B and C, for all sampling times.

| | | | Contaminant concentration in muscle (ng.g$^{-1}$ tissue ww) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $T_0$ | A | | B | | C | |
| Organic contaminant | | | | $T_{14}$ | $T_{28}$ | $T_{14}$ | $T_{28}$ | $T_{14}$ | $T_{28}$ |
| PAH | 3 - ring | Acenaphthylene | 0.91 ± 0.15 | 0.57 ± 0.10 | 0.79 ± 0.13 | 0.52 ± 0.09 | 0.80 ± 0.14 | 0.60 ± 0.10 | - |
| | | Acenaphthene | 1.82 ± 0.31 | 1.48 ± 0.25 | 1.80 ± 0.31 | 1.40 ± 0.24 | 1.70 ± 0.29 | 1.71 ± 0.29 | - |
| | | Fluorene | 1.67 ± 0.28 | 1.06 ± 0.18 | 1.89 ± 0.32 | 1.18 ± 0.20 | 1.71 ± 0.29 | 1.23 ± 0.21 | - |
| | | Phenanthrene | 3.94 ± 0.67 | 2.80 ± 0.48 | 4.21 ± 0.71 | 2.56 ± 0.44 | 3.40 ± 0.58 | 2.37 ± 0.40 | - |
| | | Anthracene | 0.27 ± 0.05 | 0.17 ± 0.03 | 0.33 ± 0.06 | 0.25 ± 0.04 | 0.33 ± 0.06 | 0.25 ± 0.04 | - |
| | 4 - ring | Fluoranthene | 1.66 ± 0.28 | 1.11 ± 0.19 | 1.68 ± 0.29 | 1.48 ± 0.25 | 1.86 ± 0.32 | 1.44 ± 0.24 | - |
| | | Pyrene | 1.58 ± 0.27 | 1.09 ± 0.19 | 1.54 ± 0.26 | 1.32 ± 0.22 | 1.66 ± 0.28 | 1.40 ± 0.24 | - |
| | | Benz[a]anthracene | 0.19 ± 0.03 | 0.12 ± 0.02 | 0.21 ± 0.04 | 0.15 ± 0.03 | 0.15 ± 0.02 | 0.11 ± 0.02 | - |
| | | Chrysene | 0.65 ± 0.11 | 0.39 ± 0.07 | 3.88 ± 0.66 | 0.44 ± 0.07 | 0.51 ± 0.09 | 0.37 ± 0.06 | - |
| | 5 - ring | Benzo[b]fluoranthrene | 0.60 ± 0.10 | 0.44 ± 0.07 | 0.56 ± 0.10 | 0.52 ± 0.09 | 0.62 ± 0.11 | 0.47 ± 0.08 | - |
| | | Benzo[k]fluoranthrene | 0.29 ± 0.05 | 0.19 ± 0.03 | 0.29 ± 0.05 | 0.25 ± 0.04 | 0.35 ± 0.06 | 0.23 ± 0.04 | - |
| | | Benzo[e]pyrene | 0.22 ± 0.04 | 0.16 ± 0.03 | 0.21 ± 0.04 | 0.19 ± 0.03 | 0.23 ± 0.04 | 0.19 ± 0.03 | - |
| | | Benzo[a]pyrene | 0.26 ± 0.04 | 0.27 ± 0.05 | 0.38 ± 0.06 | 0.29 ± 0.05 | 0.33 ± 0.06 | 0.16 ± 0.03 | - |
| | | Perylene | 0.27 ± 0.05 | 0.29 ± 0.05 | 0.30 ± 0.05 | 0.37 ± 0.06 | 0.22 ± 0.04 | 0.58 ± 0.10 | - |
| | | Dibenzo[a,h]anthracene | < d.l. | < d.l. | < d.l. | < d.l. | 0.00 ± 0.00 | < d.l. | - |
| | 6 - ring | Indeno[1,2,3-cd]pyrene | < d.l. | < d.l. | < d.l. | < d.l. | 0.00 ± 0.00 | < d.l. | - |
| | | Benzo[g,h,i]perylene | < d.l. | < d.l. | < d.l. | < d.l. | 0.00 ± 0.00 | < d.l. | - |
| | | **tPAH** | **14.32 ± 2.43** | **10.14 ± 1.72** | **18.08 ± 3.07** | **10.93 ± 1.86** | **13.87 ± 2.36** | **11.11 ± 1.89** | - |
| PCB | Trichlorinated | CB 18 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.04 ± 0.01 | 0.16 ± 0.03 | - | 0.12 ± 0.02 | - |
| | | CB 26 | < d.l. | 0.02 ± 0.00 | 0.03 ± 0.01 | 0.02 ± 0.00 | - | 0.32 ± 0.05 | - |
| | | CB 31 | 0.21 ± 0.04 | 0.43 ± 0.07 | 0.28 ± 0.05 | 1.64 ± 0.28 | - | 0.42 ± 0.07 | - |
| | Tetrachlorinated | CB 44 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.07 ± 0.01 | 0.12 ± 0.02 | - | 0.18 ± 0.03 | - |
| | | CB 49 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.06 ± 0.01 | - | 0.11 ± 0.02 | - |
| | | CB 52 | 0.07 ± 0.01 | 0.54 ± 0.09 | 0.09 ± 0.02 | 0.26 ± 0.04 | - | 0.24 ± 0.04 | - |
| | Pentachlorinated | CB 101 | 0.22 ± 0.04 | 0.14 ± 0.02 | 0.19 ± 0.03 | 0.25 ± 0.04 | - | 0.53 ± 0.09 | - |
| | | CB 105 | 0.11 ± 0.02 | 0.04 ± 0.01 | 0.19 ± 0.03 | 0.09 ± 0.01 | - | 0.14 ± 0.02 | - |
| | | CB 118 | 0.02 ± 0.00 | 0.26 ± 0.04 | 0.03 ± 0.01 | 0.41 ± 0.07 | - | 0.70 ± 0.12 | - |
| | Hexachlorinated | CB 128 | 0.07 ± 0.01 | 0.36 ± 0.06 | 0.05 ± 0.01 | 0.07 ± 0.01 | - | 0.07 ± 0.01 | - |
| | | CB 138 | 0.64 ± 0.11 | 0.31 ± 0.05 | 0.59 ± 0.10 | 0.53 ± 0.09 | - | 0.44 ± 0.07 | - |
| | | CB 149 | 0.68 ± 0.12 | 0.16 ± 0.03 | 1.58 ± 0.27 | 0.29 ± 0.05 | - | 0.49 ± 0.08 | - |
| | | CB 151 | 0.10 ± 0.02 | 0.06 ± 0.01 | 0.07 ± 0.01 | 0.13 ± 0.02 | - | 0.22 ± 0.04 | - |
| | | CB 153 | 0.64 ± 0.11 | 0.34 ± 0.06 | < d.l. | 0.61 ± 0.10 | - | 1.02 ± 0.17 | - |
| | Heptachlorinated | CB 170 | 0.05 ± 0.01 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.07 ± 0.01 | - | < d.l. | - |
| | | CB 180 | 0.21 ± 0.04 | 0.11 ± 0.02 | 0.13 ± 0.02 | 0.21 ± 0.04 | - | 0.24 ± 0.04 | - |
| | | CB 187 | 0.32 ± 0.05 | 0.18 ± 0.03 | 0.21 ± 0.04 | 0.33 ± 0.06 | - | 0.51 ± 0.09 | - |
| | | CB 194 | < d.l. | < d.l. | 0.00 ± 0.00 | < d.l. | - | < d.l. | - |
| | | **tPCB** | **3.45 ± 0.59** | **3.06 ± 0.52** | **3.66 ± 0.62** | **5.25 ± 0.89** | - | **5.75 ± 0.98** | - |
| DDT | | *pp*'DDE | 1.31 ± 0.22 | 0.77 ± 0.13 | 0.90 ± 0.15 | 1.36 ± 0.23 | - | 2.58 ± 0.44 | - |
| | | *pp*'DDD | 1.33 ± 0.23 | 0.43 ± 0.07 | 0.65 ± 0.11 | 0.29 ± 0.05 | - | 3.27 ± 0.56 | - |
| | | *pp*'DDT | 3.75 ± 0.64 | 0.28 ± 0.05 | 7.98 ± 1.36 | 0.08 ± 0.01 | - | < d.l. | - |
| | | **tDDT** | **6.39 ± 1.09** | **1.48 ± 0.25** | **9.53 ± 1.62** | **1.73 ± 0.29** | - | **5.85 ± 0.99** | - |

[< d.l.], below detection limit; [-], unavailable data. Ranges indicate the standard error.

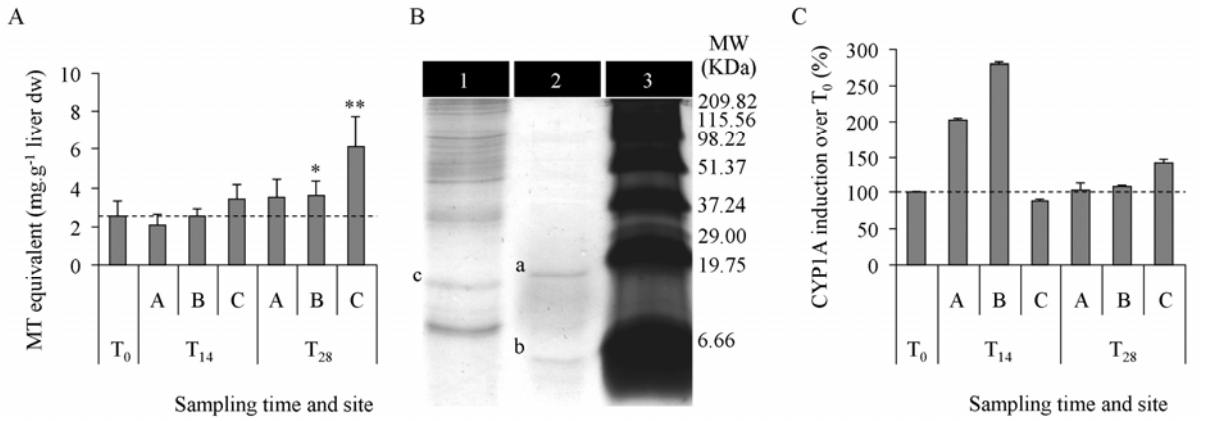



**Fig. 3.2.4.** Results from biomarker analyses. A) Average MT concentration in the liver of exposed individuals (*n* = 12 fish per treatment and sampling time). * and ** represent significant differences from $T_0$ (dashed line) $p < 0.1$ and $p < 0.01$, respectively (Student's *t*-test on GLM coefficients). Error bars represent 95% confidence intervals. B) SDS-PAGE confirmation of the presence of a MT-compatible band in *S. senegalensis* liver. Lanes: 1-Heat-treated cytosol from a $T_{28}$-collected individual (test C), 2-rabbit MT standard, 3-Broadrange protein ladder. a, b Dimeric and monomeric forms of rabbit MT, respectively, c dimeric form of *S. senegalensis* MT. MW: protein molecular weight. C) CYP1A induction in pooled liver samples of exposed individuals over $T_0$ (dashed line). Error bars represent the standard error.

## 4. Discussion and conclusions

Sediment metals were found to be in general accordance with contamination levels previously assessed in the estuary (Caeiro et al., 2005), although a moderate decline in metals can be noticed, from previous to current data, and organic contamination analysis reveals a very pronounced decrease in tPAHs for all stations (up to tenfold), the inverse being observed for tPCBs (Neuparth et al., 2005) and tDDTs in sediment B (Caeiro et al., 2009). Globally, the observed sediment contamination profiles reflect the expected distribution and sources of toxicants in the area Bioavailability of sediment contaminants was most probably enhanced by sediment disaggregation and resuspension caused by sediment collection, assay set-up and fish activity. This may explain why bioaccumulation of metals was considerably higher at the assays mid-term rather than at its terminus since a decrease in suspended matter was observed during the experiment. Metals may have been released from suspended matter and therefore made available to be absorbed through gills and other epithelia in contact with water, accumulated in the liver and then gradually eliminated when uptake decreased. Bioaccumulation of organic contaminants does not so evidently reflect this pattern but it is clearly related to sediment contamination, especially regarding PCBs and DDT metabolites (*pp*'DDD and *pp*'DDE) in sediment C-tested fish (but not *pp*'DDT itself, showing that active metabolism of the toxicant occurred). Regarding PAHs, although the best represented class of organic contaminants in the sediments, no obvious bioaccumulation occurred. Flatfish, however, have already been described to rapidly catabolize PAHs (Varanasi and Gmur 1981).

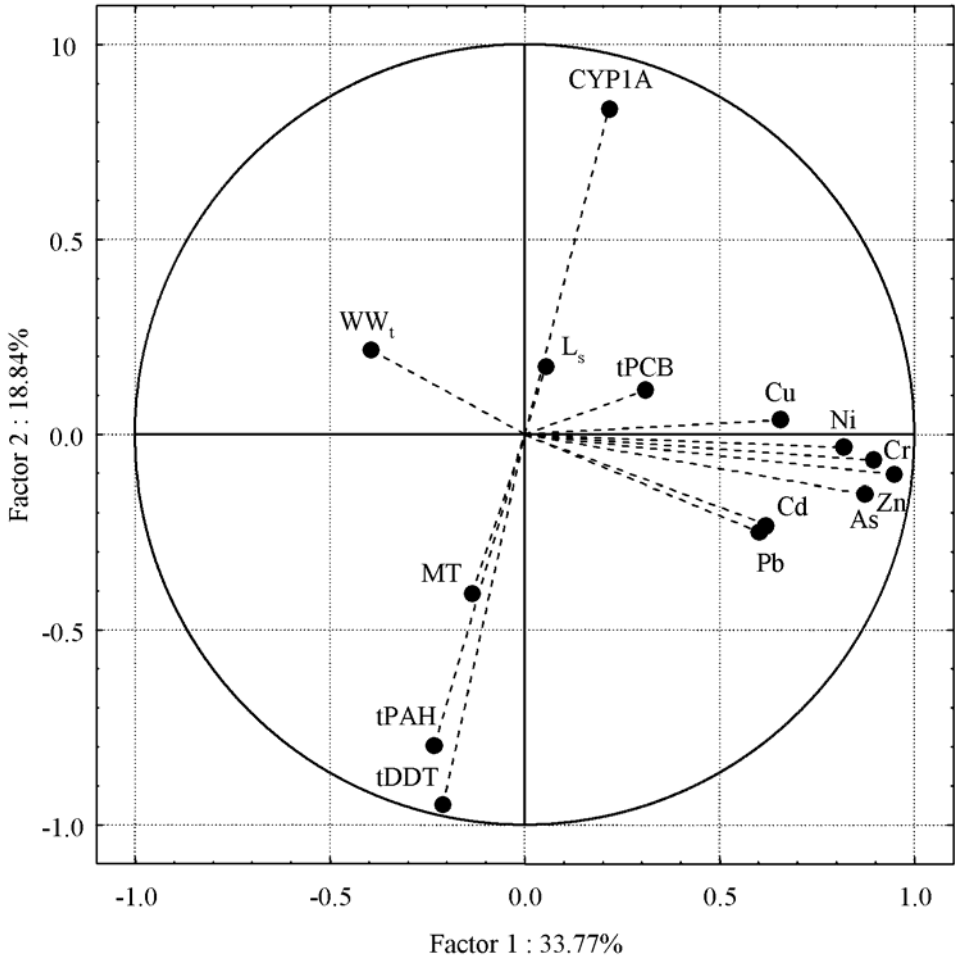



**Fig. 3.2.5.** PCA scatterplot for all biological variables (Factor 1 Eigenvalue = 4.73, Factor 2 Eigenvalue = 2.64).

**Table 3.2.3.** Results from GLM ANOVA based on analysis of deviance of the MT induction model. Only the most significant model components (explanatory variables) are presented.

| **Model component** | **$\chi^2$ test *p*** | **Coefficient type** |
|---|---|---|
| Cr in liver | < 0.05 | negative |
| Zn in liver | < 0.05 | positive |
| tPAH in muscle | 0.06 | negative |
| tDDT in muscle | < 0.05 | positive |
| $ww_t$ | 0.09 | positive |
| $ww_t$:$L_s$ | 0.08 | negative |

Even at low concentrations, metals in sediment C accumulated in the livers of tested fish. This result confirms that contaminant bioavailability (metallic and organic) was further enhanced in tests with this sediment. Together with high FF and TOM, which may have acted as a trap for contaminants

(Caccia et al., 2003), low Eh mostly likely increased bioavailability upon resuspension (Caetano et al., 2003; Eggleton and Thomas 2004), thus contributing for the higher toxicity (chronic and acute) of sediment C. The combination of FF and TOM with resuspension during the assays with natural sediments as already been detected as a probable cause of metal release from sediments, consequently enhancing metal uptake and MT response (Costa et al., 2008b). Atkinson et al. (2007) demonstrated that physical mixing and bioturbation were more responsible for the enhancement of metal availability than changes in pH, $DO_2$, or even salinity, and were furthermore responsible for maintaining high bioavailability. Other research shows that organic contaminants have more complex desorption processes (e.g. due to hydrophobicity), still enhanced by turbation (Vale et al., 1998; Hellou et al., 2005). This information supports the presented data and confirms the importance of assay conditions during sediment tests.

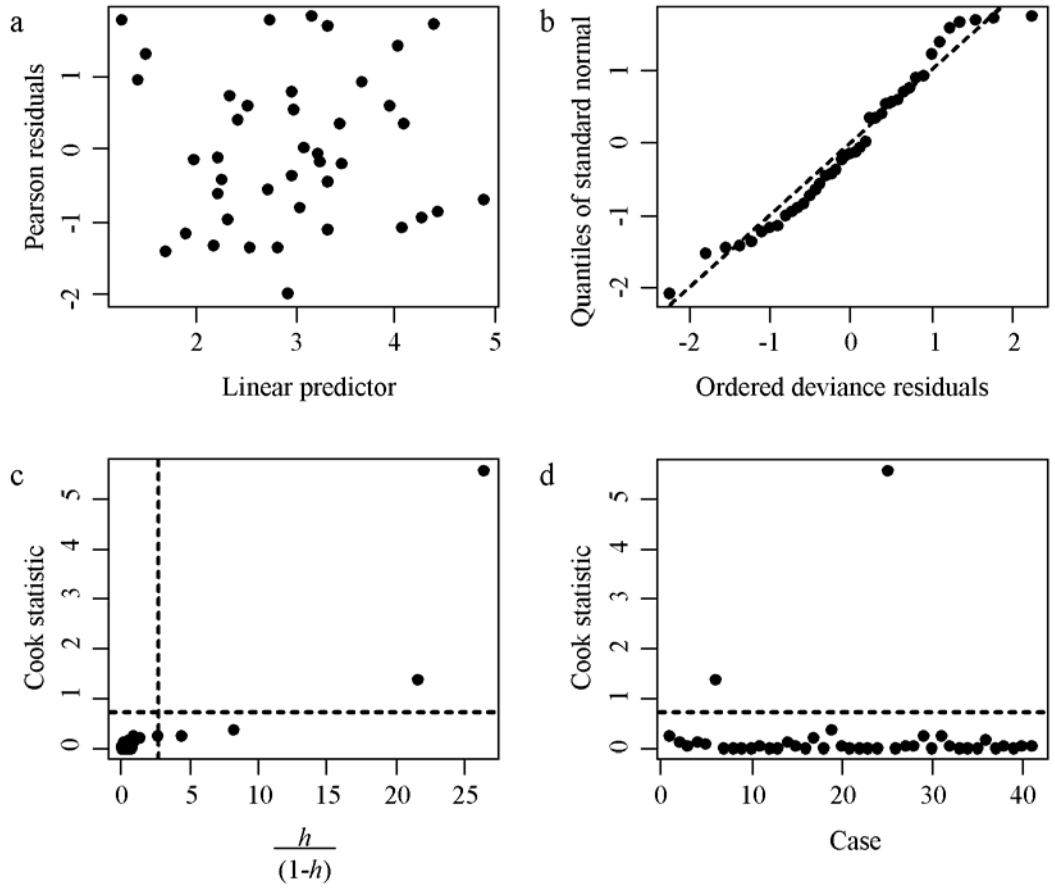



**Fig. 3.2.6.** Validation of GLM assumptions for the MT induction model. a) random scatter of Pearson residuals; b) normality of residuals; c) and d) Cook's statistic *h* results, showing that only a very reduced number of cases may cause estimate biasing.

Our findings demonstrate that MT induction cannot be exclusively explained by metals in the sediment or in the organism. MT induction is known to be modulated by both metals and organic contaminants but previous findings are sometimes contradictory. Simultaneous exposure to Cu and benzo[a]pyrene (B[a]P), was found to greatly increase MT induction in fish liver even when exposure to the individual contaminants reduced MT expression (Roméo et al., 1997). On the other hand, MT

induction caused by a mixture of metals (Cd, Cu, Pb and Zn) has been found to be suppressed by PAHs (Risso-de Faverney et al., 2000). MT induction may also be influenced by various stress--inducing conditions followed by exposure to strong MT-inducing metals. Sampaio et al. (2008) found MT induction to be modulated in fish by simultaneous exposure to moderate levels of Cu and hypoxia (but not significantly by each effect alone), accompanied by an enhancement in superoxide dismutase (SOD) activity, which should reflect an increase in ROS. In fact, the importance of MTs to scavenge oxidative radicals has already been demonstrated in fish and other aquatic organisms, appearing to be negatively correlated to the amount of metal thiolates formed, presumably due to the availability of MT thiols to bind to ROS or ROS-related compounds (Buico et al., 2008). It is thus possible that oxidative stress resulting from the catabolism of organic compounds, and not metal in liver alone, was responsible for the very significant increase in liver MT observed in C-tested individuals. According to our findings, accumulated metals play different roles in MT induction: while Cd does not appear to be linked to MT induction (likely due to its low levels in tested sediments), Cr and Zn (well represented in sediments, especially in B) have been found very significantly related to MT induction but with opposite effects. Although Cr alone is capable to induce MT synthesis in fish liver (Roberts and Oris, 2004) it is known to have an antagonist effect in MT induction in the presence of high MT--affinity metals like Cd and Zn (Majumder et al., 2003). MT induction in fish is also known to be mediated by other variables such as age, size and sex (Hamza-Chaffai et al., 1995). In the present work, the age factor can be excluded from analysis since animals belong to the same cohort (as well as sex, due to immatureness of individuals). Our results suggest that fish biomass may have incremented MT synthesis. On the other hand, a negative effect caused by the interaction between $L_s$ and $ww_t$ suggests that MT downregulation may occur when the expected linear relationship between body length and size is not met. In fact, only a moderate correlation between $L_s$ and $ww_t$ was observed (Spearman's $R = 0.69$, $p < 0.01$). This data confirms the relevance of intraspecific variability in MT response.

Some authors have argued that caution is mandatory when MT induction is considered as a biomarker of metal contamination in sediment biomonitoring, especially when low levels of strong inducers (like Cd) are involved (Mouneyrac et al., 2002). This statement is validated when our results are compared to those obtained by Jiménez-Tenorio et al. (2007), who found MT induction in *S. senegalensis* liver perfectly linked to sediment metals but with these (especially Cd and Zn) having been found to be in a tenfold order of magnitude relatively to concentrations in the sediments here described, with organic contaminants being found only in trace concentrations. The present results show that MT induction, although little related to metal exposure, can be successfully employed even in tests with moderately metal-contaminated sediments at least as a stress response, a role for fish MT that has already been proposed by other authors, e.g. Viarengo et al. (1999).

It is long known that metals and metalloids are CYP1A suppressors, even in presence of known strong CYP1A inducers (Brüschweiler et al., 1996; Vakharia et al., 2001; Spink et al., 2002). Although many details on the mechanism are still lacking, it is generally recognized that one of the

most important causes of CYP1A suppression is a metal- or metalloid-caused induction of the hemeoxygenase enzyme, enhancing degradation of haeme groups and therefore reducing its availability as the prosthetic group of CYP1A (Spink et al., 2002). This inhibitor effect may actually decrease short-term PAH toxicity since PAH degradation involves formation of the highly toxic activated PAH forms (e.g. PAH *o*-quinones and diol epoxides) and ROS as by-products of cytochrome activity (e.g. Flowers-Geary et al., 1996), thus contributing to explain why sediment B was responsible for much reduced lethality when compared to C. The absence of CYP1A induction in sediment C--tested fish sampled at $T_{14}$ is only partially explained by cytochrome suppression caused by metals but still, the opposite trend between accumulated PAHs (and DDTs) and CYP1A induction confirms the cytochrome's role in the metabolism of organic contaminants. It should not be discarded, nevertheless, that a rapid organic contaminant uptake and catabolism at the beginning of the assay with sediment C may have also led to depletion in accumulated compounds and consequently contributed to a reduction in CYP1A induction from $T_0$ to $T_{14}$ and an increase in activated PAHs and ROS.

Downregulation of CYP1A has already been documented as a direct result of ROS activity (Morel and Barouki, 1998) and exposure to PCBs (Celander et al., 1996). Although not yet well understood, it is known that tetra- and penta-chlorinated PCBs may induce CYP1A protein synthesis but suppress its activity in fish liver in a dose-dependent manner, causing a significant increase in ROS (Gooch et al., 1989; Schlezinger and Stegeman, 2001). Since tetra- and penta-chlorinated PCBs are relatively well represented in sediment C (approximately threefold more contaminated with these PCBs than B and almost tenfold than A) formation of ROS and PCB bioaccumulation in livers may have resulted in a diminished CYP1A induction.

The results show that sediment parameters may influence toxicity by determining bioavailability. Furthermore, the laboratorial assays were proven to enhance bioavailability by a conjunction of sediment physico-chemical characteristics and resuspension, allowing an estimate of increasing toxicity to biota when sediment-disturbing events such as dredgings, heavy seasonal runoffs or storms affect contaminated estuarine areas. The present work demonstrated that toxicity of sediment contaminants to young benthic fish is influenced by synergistic and antagonistic processes that result from exposure to a mixture of xenobiotics. The biomarkers and bioaccumulation analyses were successfully integrated and allowed assessment of cause-effect relationships between contaminants and toxicity and, most importantly, provided a valuable insight on what happens in the fuzzy area where actual toxicity results from the superimposition of contaminants in complex matrices like estuarine sediments.

**Acknowledgments**

This work was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB

57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors would also like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and RNES (Reserva Natural do Estuário do Sado) for the logistic support and P. Pousão (Estação Piloto de Piscicultura, INRB/IPIMAR-Cripsul) for supplying the fish used in the present work.

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## 3.3. Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: a weighted indices approach[†]


### Abstract

Young juvenile *Solea senegalensis* were exposed to three sediments with distinct contamination profiles collected from a Portuguese estuary subjected to anthropogenic sources of contamination (the Sado Estuary, western Portugal). Sediments were surveyed for metals (cadmium, chromium, copper, nickel, lead and zinc), a metalloid (arsenic) and organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls and a pesticide, dichloro-diphenyl-trichloroethane plus main metabolites), as well as total organic matter, redox potential and particle fine fraction. The fish were exposed to freshly collected sediments in a 28-day laboratorial assay and collected for histological analyses at days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$). Individual weighted histopathological indices were obtained, based on presence/absence data of eight and nine liver and gill pathologies, respectively, and on their biological significance. Although livers sustained more severe lesions, the sediments essentially contaminated by organic substances caused more damage to both organs than the sediments contaminated by both metallic and organic contaminants, suggesting a possible synergistic effect. Correlation analyses showed that some alterations are linked, forming distinctive histopathological patterns that are in accordance with the severity of lesions and sediment characteristics. The presence of large eosinophilic bodies in liver and degeneration of mucous cells in gills (a first-time described alteration) were some of the most noticeable alterations observed and were related to sediment organic contaminants. Body size has been found to be negatively correlated with histopathological damage in livers following longer term exposures. It is concluded that histopathological indices provide reliable and discriminatory data even when biomonitoring as complex media as natural sediments. It is also concluded that the effects of contamination may result not only from toxicant concentrations but also from their interactions, relative potency and sediment characteristics that ultimately determine bioavailability.



### Key-words

*Solea senegalensis*; Histopathological indices; Bioassays; Sado Estuary; Sediment; Metallic and organic contaminants


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[†] Costa et al. (2009). *Aquat. Toxicol.* **92**, 202-212 (doi:10.1016/j.aquatox.2008.12.009).

## 1. Introduction

An increasing amount of research is now incorporating histopathological biomarkers in practical ecological risk assessment methodologies. Histopathological analysis has already been tested and proposed as an efficient and sensitive tool to the monitoring of fish health and environmental pollution in natural water bodies (Teh et al., 1997; Handy et al., 2002; Wester et al., 2002; Stentiford et al., 2003). The growing number of studies on histopathological biomarkers is linked to the notion that they reflect fish health more realistically than biochemical biomarkers and can thus be better extrapolated to community- and ecosystem-level effects of toxicity (Au, 2004).

Classical, essentially qualitative histopathological approaches have provided vital information on the description of histological lesions and alterations in field-collected or tested aquatic organisms (e.g. Baumann, 1985; Köhler, 1990). Nevertheless, the absence of numerical data makes it difficult to establish cause-effect relationships between pathology and contamination patterns and to assess the significance of the differences between surveyed groups. For such reason, current research on histopathological traits of exposed animals is now focusing on histopathological indices to provide numerical data based on a semi-quantitative approach. Some of these approaches have successfully employed multivariate statistics using lesion frequency indices to compare contaminated sites in biomonitoring studies (e.g. DelValls et al., 1998; Riba et al., 2004, 2005). Other authors have demonstrated the usefulness of semi-quantitative ranking indices based on lesion progression in several fish organs, with the advantage of providing individual indices (e.g. Schwaiger, 2001; Van Dyk et al., 2007; Triebskorn et al., 2008).

One of the most important difficulties of histopathological studies in fish relates to the lack of specificity of lesions and alterations towards a contaminant or class of contaminants, which greatly impairs cause-effect assessments when multiple toxicants are involved. On the other hand, tissue-level pathologies are by far better described in human biomedicine than in ichthyology and discrepancies in terminology and identification of lesions often arise. In an attempt to solve this issue, research is being performed in order to provide guidelines on the histopathological endpoints of exposure to xenobiotics (e.g. Koehler, 2004). Another endpoint under development concerns the actual biological significance of the analyzed lesions. Some authors now propose that condition indices should consider the relative importance of lesions since some alterations may imply greater injury to an organ than others. Weighted indices have been developed in order to fulfil this gap by attributing an ordinal-ranked value to a specific lesion according to its impact to the fish (Bernet et al., 1999).

The choice of the target organisms is also a critical factor in environmental monitoring. Due to their increased sensitivity to environmental contaminants and severity of effects on development, as well as the consequences to ecosystems and marine resources, many toxicological studies have focused on early life stage fish (Rolland, 2000). Histopathological analyses have, for instance, been successful in the assessment of the effects of organochlorine pesticides on organ development in fish larvae (Oliva et al., 2008) and hepatic lesions in juvenile fish exposed to PAHs, PCBs and

organochlorines (Metcalfe et al., 1990). On the other hand, flatfish (including *Solea senegalensis*) have been successfully employed in field surveys (Simpson et al., 2000; Stentiford et al., 2003) or laboratorial exposures to sediments (Riba et al., 2004, 2005; Jiménez-Tenorio et al., 2007; Costa et al., 2008) and waterborne xenobiotics (Arellano et al., 1999; Grinwis et al., 2000).

The Senegalese sole, *S. senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae), is a common flatfish in the Sado Estuary, where it is an important resource, or at least a valuable by-catch, for local fisheries. This benthic fish inhabits estuaries especially as breeding and nursing grounds, occupying sandy or muddy bottoms where it feeds on small invertebrates (Cabral and Costa, 1999; Cabral, 2000). It is a cosmopolitan species on the Atlantic coast of the Iberian Peninsula and an important aquaculture species (Dinis et al., 1999). Its ecological characteristics and ready availability (either from the field or from mariculture facilities) contribute to the species' potential as a sentinel organism for the biomonitoring of estuarine sediment contamination.

The Sado estuarine basin (Western Portugal) is a large confined coastal area subjected to various sources of anthropogenic contamination, ranging from the urban effluents from the city of Setúbal to industrial discharges from its dense heavy-industry belt. Runoffs from the extensive agricultural grounds located upstream also contribute to the transport of xenobiotics (such as pesticides and fertilizers) to the Sado basin. The estuary is an important port area and is frequently subjected to dredging to expand wharfs and to maintain navigation channels. Aquaculture and fisheries are also very important activities in the area, as well as tourism, and part of the estuary is classified as a natural reserve area. The conflict between exploitation and the need to safeguard environmental quality enhances the importance of biomonitoring studies in the estuary.

The main goals of the present work were to: (i) assess lesions and alterations on gills and livers of juvenile *S. senegalensis* exposed to sediments from three distinct stations of the Sado Estuary; (ii) derive weighted histopathological condition indices; and (iii) investigate the relation between indices, lesion frequencies and sediment contaminants using a wide range of statistical analyses.

## 2. Materials and methods

### *2.1. Experimental assay*

The tested sediments were collected with a Petit Ponar grab on November 2006 from three distinct sites of the Sado Estuary (Fig. 3.3.1). Site A (the least contaminated) is the closest to a natural reserve area, the farthest from possible contamination sources and has the shortest water residence time. Sites B and C, located off Setúbal's harbour or the city's industrial belt, respectively, are potentially the most contaminated, although with different levels of contamination by metallic and organic toxicants. After collection, sediments were homogenized, transported under controlled temperature to the laboratory and were subdivided and frozen for analyses (refer to following section) or preserved at 4

ºC for no longer than 5 days before the beginning of tests. For simplification purposes, exposure to the three sediments is throughout referred to as sediment tests A, B and C.

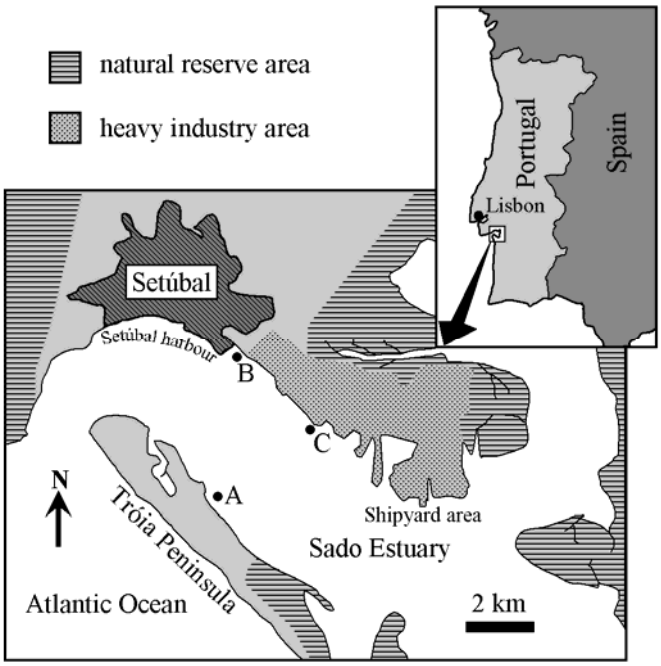


**Fig. 3.3.1.** Map of the study area showing the three sediment collection sites [A, B and C](•).

The experimental 28-day assay consisted of a closed-system recirculation arrangement of 15 L-capacity polyvinyl tanks with smooth edges to which 2 L of sediment and 12 L of clean seawater were allocated. The assay was performed in duplicate. Sediments (occupying a surface of ≈ 525 $cm^2$ in the tanks) were allowed to settle for 48 h before the beginning of the assay. Aeration was constant and water flow was adjusted in order to eliminate hydrodynamically driven sediment resuspension. A weekly water change (25% of total water volume) was performed in order to mimic and keep constant the animals' rearing conditions while ensuring minimal removal of potential waterborne contaminants or suspended particles and minimal stress to the fish in the test tanks. Water parameters were monitored weekly, just prior to water changes and were found to be the same as in rearing: pH 7.9 ± 0.2, salinity = 33 ± 1 $g.L^{-1}$, temperature = 18 ± 1 ºC, dissolved $O_2$ ranged between 40 and 45% and total ammonia within 2 - 4 $mg.L^{-1}$. Photoperiod was set at 12:12 h light:dark.

Twenty four randomly selected juvenile hatchery-brood and laboratory-reared *S. senegalensis* (69 ± 6 mm standard length), all from the same cohort, were allocated to each tank. Fish were fed daily with M2 grade commercial fish pellets (Aquasoja, Ovar, Portugal) throughout the assay. Twelve individuals (six per replicate) from each sediment test were collected per sampling time, scheduled for days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$) and immediately processed for histological analyses. $T_0$ animals consisted of twelve individuals collected directly from the rearing tanks.

*2.2. Sediment analyses*

The redox potential (Eh) of the sediments was determined immediately after collection using an Orion model 20A apparatus equipped with a H3131 Ag/AgCl reference electrode. Sediments were characterized for total organic matter (TOM) by complete ignition at 500 ± 50 ºC. Fine fraction (FF, particle size < 63 μm) was determined by hydraulic sieving after removal of organic matter with $H_2O_2$, washing and disaggregation in pyrophosphate. Fine fraction and TOM are described as a percentage relatively to sediment dry weight (dw).

Trace elements were quantified from dried samples completely mineralized with a mixture of acids (6 mL HF 40%, v/v to which was added 1 mL of the mixture 36% HCl plus 60%$HNO_3$ 3:1 v/v) for 1 h at 100 ºC in closed Teflon vials, evaporated to dryness and redissolved in HNO3 before elution in Milli-Q grade ultrapure water (Caetano et al., 2007). Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) were quantified by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series spectrometer. MESS-2 (NRC, Canada), PACS-2 (National Research Council, Canada) and MAG-1 (USGS, USA) reference sediments were analyzed by the same procedure to validate the procedure and the obtained metal concentrations were found to be within the certified range. Results are given in $\mu g.g^{-1}$ sediment dw.

Sediment PAHs were analyzed as described by Martins et al. (2008). Briefly: dry sediment samples were spiked with surrogate standards, Soxhlet-extracted with an acetone + hexane (1:1 v/v) mixture and quantified by comparison with the retention time of standard by gas chromatography--mass spectrometry (GC-MS) using a Finnigan GCQ system. A total of seventeen 3- to 6-ring PAHs were quantified. tPAH means the sum of all individual PAHs. PCBs (18 congeners) and DDTs (*pp*'DDT plus metabolites: *pp*'DDD and *pp*'DDE) were quantified from dried sediment samples Soxhlet-extracted with *n*-hexane, fractioned in a chromatographic column and quantified by GC with electron capture detector (GC-ECD) with a Hewlett-Packard 6890 gas chromatograph (Ferreira et al., 2003). tPCB and tDDT mean the sum of all quantified PCB congeners and DDT plus metabolites, respectively. Validation was obtained by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the concentrations of surveyed organic compounds were found within the certified range. Concentrations of sediment organic contaminants are expressed as $ng.g^{-1}$ sediment dw.

*2.3. Sample preparation for histological analyses*

Animals were anaesthetized on ice after collection, measured for standard length ($L_s$) and total wet weight ($ww_t$) and euthanized by cervical sectioning. Dissection was performed immediately and samples were prepared for histological analyses essentially according to Martoja and Martoja (1967). In brief: liver samples and the first and second gill arches (from the eyed side) were excised and immediately placed in Bouin-Hollande fixative (10% v/v formaldehyde and 7% v/v acetic acid to

which picric acid was added to saturation), where they remained for 48 h (at room temperature). Samples were afterwards washed for 24 h to remove excess picric acid in a bath of distilled water (liver) or a 6% (v/v) formic acid solution in distilled water to promote decalcification (gills). Samples were afterwards dehydrated in a progressive series of ethanol dilutions and embedded in paraffin (xylene was used for intermediate impregnation). Sections (2-3 μm thick) were stained with haematoxylin and counterstained with alcoholic eosin (H&E stain) for structural analysis of gills and liver. Gill sections were also stained with Alcian blue for the detection of mucosubstances (such as mucopolysaccharides and sialomucin glycoproteins) and counterstained with nuclear fast red (AB&NFR stain). Slides were mounted with DPX resinous medium (from BDH). Slides were prepared in duplicate for each organ and staining procedure, with 6-8 sections per slide. A blind review of slides was performed at the end of analyses to confirm the accuracy in identification of histological traits. A DMLB model microscope (Leica Microsystems) was used for all analyses. Image analysis was performed with the software ImageJ (Wayne Rasband National Institute of Health, USA).

*2.4. Histopathological condition indices*

Histopathological condition indices for liver and gills were essentially adapted from Bernet et al. (1999). For each alteration an importance factor, or condition weight ($w$), was assigned, as proposed by Bernet and co-workers, based on the biological significance of the lesion, i.e. the degree in which a lesion may affect the normal functioning of a tissue or organ. Accordingly, two histopathological indices ($I$) were calculated: $I_l$ (for liver) and $I_g$ (for gills). The indices were obtained for each individual and were calculated by the simple formula:

$$I = \sum_{j=1}^{n} w_j o_j \qquad [1]$$

Where $w_j$ is the relative weight of the $j^{th}$ condition and $o_j$ a Boolean variable that assumes the values: 1 (observed) or 0 (unobserved). $n$ is the total number of pathologies analyzed in the organ. The indices are, therefore, cumulative and account for not only the number of alterations observed in each individual but also their relative importance. Only persistent pathologies within an organ were scored as observed ($o_j$ = 1), meaning that point alterations that did not qualify as representative of the overall organ condition (e.g. one necrotic cell observed in an entire liver portion or two fused lamellae in a gill arch) were disregarded, being considered as natural variations. Identification of histopathological alterations was primarily based on Hibiya (1982) and Arellano et al. (1999, 2004).

*2.5. Statistical analyses*

Statistics were based on the individual *I* values and comprised analysis of variance by means of the *F*-test (parametric) to assess overall differences between tests. Pairwise comparisons were obtained with the Tukey's Honest Significant Differences test (HSD test, parametric). Parametric statistics were employed after validation of the homogeneity of variances (through the Levene's test) and normality of data (by the Kolmogoroff-Smirnoff test). Non-parametric statistics (Kruskall-Wallis ANOVA by ranks *H* and the Mann-Whitney *U* test) were performed when at least one of these assumptions was not met. Cluster analysis was based on correlation matrices by computing the Pearson's *r* statistic. Pairwise correlations were obtained through the Spearman's rank-order correlation *R*. The significance level was set at $\alpha = 0.05$. Statistical analysis was conducted according to Sheskin (2000) and Zar (1998) and was performed with the Statistica software package (Statsoft Inc.).

## 3. Results

*3.1. Sediment characterization*

The sediments from the three sites exhibited distinct characteristics and contamination profiles (Table 3.3.1). Sediment A was found to be the least contaminated by both metallic and organic compounds and also the least anoxic sediment (i.e. with highest Eh). Sediment B presented the highest levels of all surveyed metallic contaminants and also showed the highest proportion of FF and TOM. Sediment C (the most anoxic) was essentially contaminated by organic compounds. Sediment B contained high levels of organic contaminants, especially PAHs. However, some of these substances are present in sediment C in much greater levels than those observed in B. One such compound is perylene which is present in sediment C at 241% of the concentrations observed in sediment B. Overall, the most significant PAHs were 4- and 5-ring compounds, representing ≈ 70% of tPAH in the three sediments. The phenanthrene/anthracene and fluoranthene/pyrene ratios were > 1 and < 10, respectively, for all sediments, which reflects the essentially pyrolytic origin (combustion-derived) of PAHs, as opposed to being of petrogenic origin [i.e. derived from fossil fuels (Budzinski et al., 1997)]. PCBs in sediment C were almost 3-fold compared to the levels found in sediment B, with penta- and hexachlorinated congeners representing more than 90% of tPCB. DDTs were the least represented organic toxicants, with the highest values being observed in sediment B, especially *pp*'DDT.

**Table 3.3.1.** General characterization of tested sediments.

| | | | Site | | |
|---|---|---|---|---|---|
| | | | **A** | **B** | **C** |
| Sediment parameters | | TOM (%) | 3 | 12 | 8 |
| | | FF (%) | 37 | 98 | 77 |
| | | Corrected Eh (mV) | -233 | -290 | -316 |
| Metallic (mg.kg$^{-1}$ sediment dw) | | As | 7.25 ± 0.15 | 27.43 ± 0.55 | 12.38 ± 0.25 |
| | | Cd | 0.04 ± 0.00 | 0.22 ± 0.00 | 0.15 ± 0.00 |
| | | Cr | 24.20 ± 0.48 | 76.33 ± 1.53 | 21.85 ± 0.44 |
| | | Cu | 22.57 ± 0.45 | 167.32 ± 3.35 | 41.18 ± 0.82 |
| | | Ni | 12.97 ± 0.26 | 33.67 ± 0.67 | 9.03 ± 0.18 |
| | | Pb | 23.70 ± 0.47 | 66.49 ± 1.33 | 45.17 ± 0.90 |
| | | Zn | 147.48 ± 2.95 | 312.23 ± 6.24 | 87.75 ± 1.76 |
| Organic (µg.kg$^{-1}$ sediment dw) | PAH | Acenaphthene | 1.41 ± 0.24 | 9.42 ± 1.60 | 4.19 ± 0.71 |
| | | Acenaphthylene | 0.24 ± 0.04 | 1.83 ± 0.31 | 1.95 ± 0.33 |
| | 3 - ring | Anthracene | 1.03 ± 0.17 | 10.60 ± 1. | 15.34 ± 2.61 |
| | | Fluorene | 1.32 ± 0.22 | 8.70 ± 1.48 | 8.03 ± 1.37 |
| | | Phenanthrene | 7.96 ± 1.35 | 50.77 ± 8.63 | 54.09 ± 9.20 |
| | | Benz[a]anthracene | 4.53 ± 0.77 | 64.60 ± 10.98 | 86.52 ± 14.71 |
| | 4 - ring | Chrysene | 2.20 ± 0.37 | 28.31 ± 4.81 | 37.19 ± 6.32 |
| | | Fluoranthene | 18.05 ± 3.07 | 170.80 ± 29.04 | 184.30 ± 31.30 |
| | | Pyrene | 14.66 ± 2.49 | 131.74 ± 22.40 | 171.39 ± 29.14 |
| | | Benzo[a]pyrene | 7.56 ± 1.28 | 69.81 ± 11.87 | 85.88 ± 14.60 |
| | | Benzo[b]fluoranthrene | 6.77 ± 1.15 | 60.86 ± 10.35 | 70.25 ± 11.94 |
| | 5 - ring | Benzo[e]pyrene | 5.12 ± 0.87 | 56.73 ± 9.64 | 62.76 ± 10.67 |
| | | Benzo[k]fluoranthrene | 4.16 ± 0.71 | 32.21 ± 5.48 | 40.18 ± 6.83 |
| | | Dibenzo[a,h]anthracene | 0.74 ± 0.13 | 7.45 ± 1.27 | 6.99 ± 1.19 |
| | | Perylene | 4.69 ± 0.80 | 86.97 ± 14.79 | 209.16 ± 35.56 |
| | 6 - ring | Benzo[g,h,i]perylene | 1.12 ± 0.19 | 39.12 ± 6.65 | 10.44 ± 1.78 |
| | | Indeno[1,2,3-cd]pyrene | 4.87 ± 0.83 | 52.44 ± 8.91 | 51.82 ± 8.81 |
| | | **tPAH** | **86.4 ± 14.7** | **882.4 ± 150.0** | **1,100.5 ± 187.1** |
| | PCB | CB 18 | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.09 ± 0.01 |
| | Trichlorinated | CB 26 | 0.05 ± 0.01 | 0.06 ± 0.01 | 0.09 ± 0.01 |
| | | CB 31 | 0.64 ± 0.11 | 0.19 ± 0.03 | < d.l. |
| | | CB 44 | 0.05 ± 0.01 | 0.38 ± 0.06 | < d.l. |
| | Tetrachlorinated | CB 49 | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.36 ± 0.06 |
| | | CB 52 | 0.05 ± 0.01 | 0.12 ± 0.02 | 0.45 ± 0.08 |
| | | CB 101 | 0.04 ± 0.01 | 0.23 ± 0.04 | 1.18 ± 0.20 |
| | Pentachlorinated | CB 105 | 0.03 ± 0.01 | 0.22 ± 0.04 | 0.66 ± 0.11 |
| | | CB 118 | < d.l. | 1.04 ± 0.18 | 4.92 ± 0.84 |
| | | CB 128 | 0.01 ± 0.00 | 0.08 ± 0.01 | < d.l. |
| | | CB 138 | 0.12 ± 0.02 | 0.68 ± 0.12 | 2.68 ± 0.46 |
| | Hexachlorinated | CB 149 | 0.11 ± 0.02 | < d.l. | < d.l. |
| | | CB 151 | 0.05 ± 0.01 | 0.17 ± 0.03 | 1.15 ± 0.20 |
| | | CB 153 | 0.14 ± 0.02 | 0.64 ± 0.11 | 3.39 ± 0.58 |
| | | CB 170 | 0.07 ± 0.01 | 0.27 ± 0.05 | < d.l. |
| | Heptachlorinated | CB 180 | 0.21 ± 0.04 | 0.61 ± 0.10 | < d.l. |
| | | CB 187 | 0.20 ± 0.03 | 0.72 ± 0.12 | < d.l. |
| | | CB 194 | 0.03 ± 0.00 | 0.07 ± 0.01 | 0.38 ± 0.06 |
| | | **tPCB** | **1.87 ± 0.32** | **5.64 ± 0.96** | **15.34 ± 2.61** |
| | DDT | *pp*'DDD | 0.10 ± 0.02 | 0.28 ± 0.05 | 0.60 ± 0.10 |
| | | *pp*'DDE | 0.05 ± 0.01 | 0.27 ± 0.05 | 0.65 ± 0.11 |
| | | *pp*'DDT | 0.70 ± 0.12 | 4.39 ± 0.75 | 1.18 ± 0.20 |
| | | **tDDT** | **0.85 ± 0.14** | **4.94 ± 0.84** | **2.43 ± 0.41** |

FF, sediment fine fraction; TOM, sediment total organic matter; PAH, polycyclic aromatic hydrocarbon; tPAH, total PAH (sum of all individual PAHs); PCB, polychlorinated biphenyl; tPCB, total PCB (sum of all congeners); *pp*'DDD, 1,1-dichloro-2,2-bis[*p*-chlorophenyl]ethane; *pp*'DDE, 1,1-dichloro-2,2-bis[*p*--chlorophenyl]ethylene; *pp*'DDT, 1,1,1-trichloro-2,2-bis[*p*-chlorophenyl]ethane; tDDT, total DDT (*pp*'DDD + *pp*'DDD + *pp*'DDT); [< d.l.], below detection limit.

*3.2. Mortality and growth*

Overall mortality registered at the end of the assays was distinct between the three tests: test A caused 2% mortality whereas in tests B and C mortality was 13% and 48%, respectively. No significant differences were found between sampling times regarding fish standard length (Kruskall--Wallis $H$, $p = 0.84$) and total wet weight (Kruskall-Wallis $H$, $p = 0.97$), as well as between tests (Kruskall-Wallis $H$, $p = 0.96$ and $p = 0.70$, for length and weight, respectively). Both measurements were very significantly correlated (Spearman $R = 0.82$, $p < 0.01$).

*3.3. Liver histopathology*

The occurrence of lesions in the livers of fish collected at $T_0$ was low. A lesion gradient, increasing from A- to C-tested fish was clearly discernible. Lesion occurrences and severity also exhibited a tendency to increase with sampling times, for all sediment tests. Individuals collected at $T_0$ largely presented normal livers (Fig. 3.3.2A), exhibiting regular cells with a translucent, virtually unstained cytoplasm in which inclusions were absent. These clear-type hepatocytes observed in healthy livers stained with H&E should indicate good storage of glycogen (Simpson, 1992). Nuclei were observed to be of constant-size and shape, with well individualized nucleoli. Many sinusoids line the hepatic cords, branching from large venous vessels.

Foci of eosinophilic (acidophilic) hepatocellular alteration were found in exposed individuals of all tests but with an obvious increase in occurrences in B- and C-tested fish, especially at $T_{28}$. Some $T_0$ individuals also exhibited this non-specific pathology that is considered to be a pre-neoplastic lesion (Vethaak and Wester, 1996). These foci were occasionally found to be associated with proliferation and swelling of blood vessels (Fig. 3.3.2B). Altered cells were frequently observed to have intraplasmatic anomalies such as vacuolation derived from lipidosis and eosinophilic bodies (Fig. 3.3.2C).

Focal hepatic necrosis was observed in all tests at all sampling times except in $T_0$ and A-tested individuals collected at $T_{14}$. Nevertheless, the occurrence and extension of necrotic areas was more significant in B- and, especially, C-tested individuals, reaching the stage where hepatic structure was no longer discernible and tissue underwent structural rupturing (Fig. 3.3.2C). Melanomacrophages were often observed in necrotic areas. Eosinophilic bodies are cytoplasmic, well-delimited, reddish inclusions commonly observed in association with strongly damaged tissue (Fig. 3.3.2C). Under H&E stain these inclusions retain a strong red pigmentation (from eosin). The presence of eosinophilic bodies is occasionally termed hyaline degeneration. The presence of these inclusions was most prominent in C-test animals. Eosinophilic bodies appeared to be membrane delimited, ellipsoidal in shape and were present in small numbers inside the cells, usually one or two. These inclusions were variable in size. Although close to the adopted $\alpha$ no statistical differences (Mann-Whitney $U$, $p = 0.07$) was found between the length of the largest axles of eosinophilic bodies of $T_{14}$ and $T_{28}$ C-tested fish

(3.3 ± 1.7 µm and 5.7 ± 0.6 µm, respectively).

Foci of unspecified granulomatous lesions were occasionally observed in the livers of C-tested individuals. These lesions consisted of foci of highly degenerated tissue where melanomacrophages were discernible, without having been observed to form dense centres (Fig. 3.3.2D).

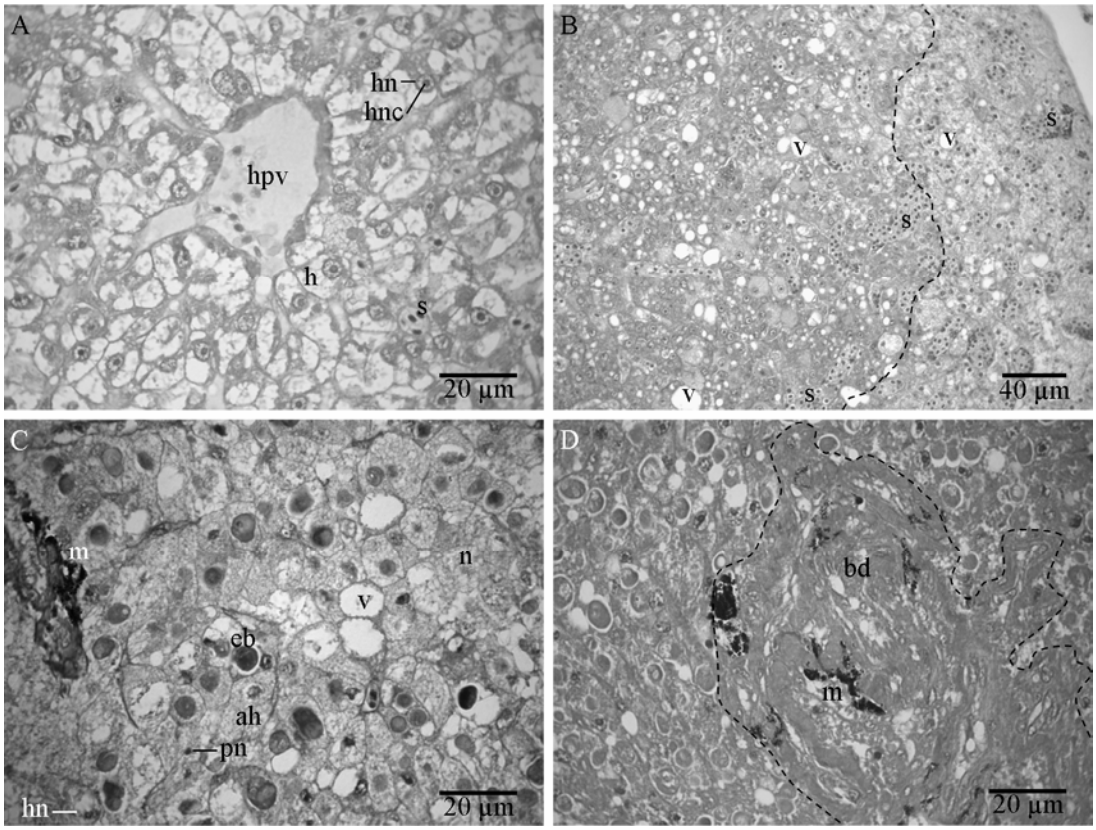


**Fig. 3.3.2** Liver sections of tested individuals (H&E). (A) Normal hepatic parenchyma of a fish collected in the beginning of the assay, exhibiting well-defined hepatocytes, polyedric in shape. (h) hepatocyte; (hn) hepatocyte nucleus; (hnc) nucleolus; (hpv) hepatic portal vein branch with erythrocytes; (s) transversally sectioned sinusoid. (B) Extensive lipidosis causing proliferation of intracellular vacuole-like structures (v) and a large eosinophilic area (right to dashed line) in a fish exposed for 14 days to sediment B (contaminated by metallic and organic substances). Proliferation and swelling of sinusoids (s) are also evident. (C) Eosinophilic hepatocellular alteration in the liver of a fish exposed for 28 days to sediment C (essentially contaminated by organic compounds). Altered hepatocytes (ah) tend to retain eosin in the cytoplasm and to lose the typical polyedric shape. Melanomacrophages (m) at a vein are also evident, as well as necrotic foci (n), vacuoles (v) and eosinophilic bodies inside altered hepatocytes (eb). Many altered cells exhibit nuclear pleomorphisms [pyknotic (pn) and hypertrophied (hn) nuclei]. (D) Granulomatous lesion in the liver of a fish exposed to sediment C for 28 days. The lesion (inside the dashed line) is located around a regressed bile duct (bd) and infiltrates the highly damaged surrounding tissue. Melanomacrophages (m) can be observed inside the lesion.

### *3.4. Gill histopathology*

As opposed to what was observed in livers, more than half of $T_0$ individuals (i.e. collected from the rearing tanks) showed moderate gill damage (Fig. 3.3.3A). Individuals tested with sediments

B and C were found to suffer the most severe lesions. Sediment particles were not observed on lamellae and interlamellar spaces. No ecto- or endoparasites were observed.

A moderate hyperplasia of interlamellar epithelial cells was often observed, occasionally originating foci of lamellar fusion (but not rod-shaped filaments), particularly in C-tested individuals (Fig. 3.3.3B). Chloride cell hypertrophy was a also frequent lesion in B and C-tested individuals (Fig. 3.3.3B). This type of damage provided gill epithelia with a vacuolated appearance since hypertrophied chloride cells enlarge and gain a vacuole-like appearance, indicating a possible fluid retention. The mean length of the largest diameter of normal chloride cells was 9.2 ± 1.1 µm (of $T_0$ fish) and that of hypertrophied was 14.7 ± 4.2 µm (of B- and C-tested individuals). The difference between the two forms was found to be statistically significant (Mann-Whitney $U$, $p < 0.05$). No obvious change in the number of chloride cells was observed.

The fish exposed to sediment C frequently presented circulatory disturbances, the most recurrent of which was the swelling of the apical vessels of lamellae due to blood congestion [also termed aneurysm or telangiectasia (Fig. 3.3.3C)]. In addition, several types of structural deformities in gill lamellae were observed (Fig. 3.3.3D), especially in B- and C-tested fish, and the most damaged gills often presented severe hypertrophy and shedding of squamous epithelia cells (desquamation).

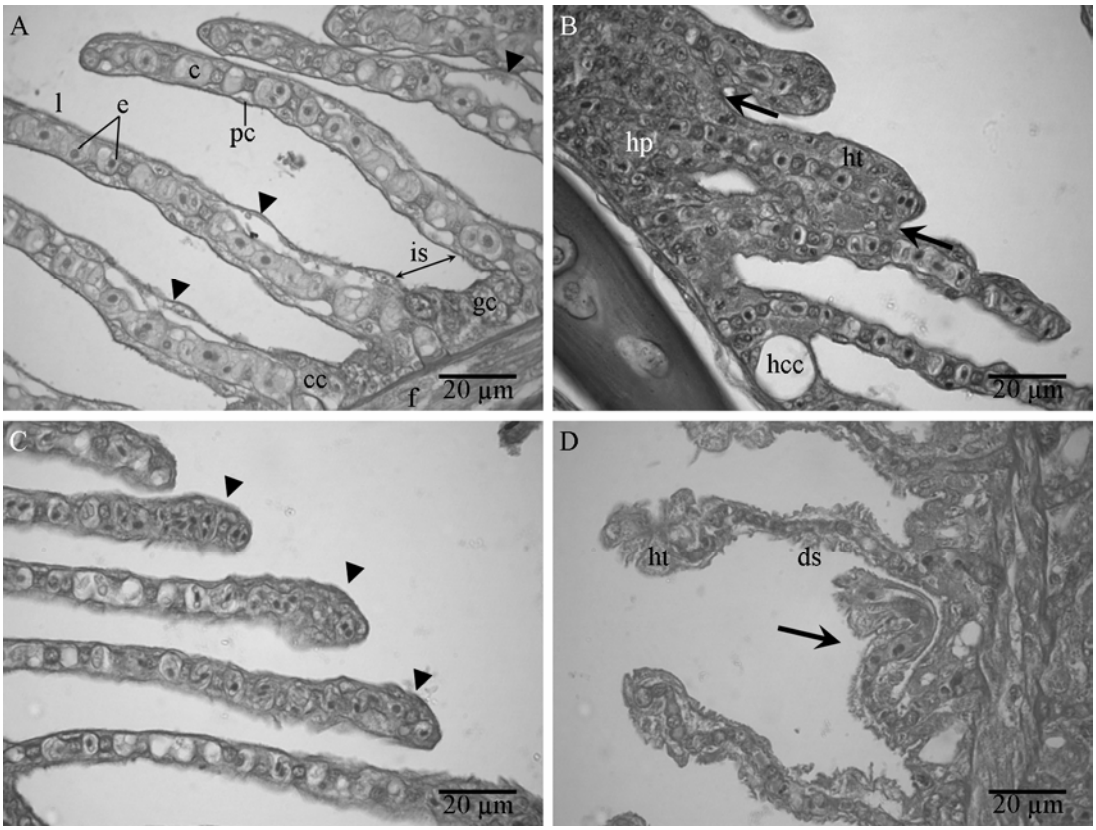


**Fig. 3.3.3.** Gill sections of tested fish (H&E). (A) Gill from an individual collected in the beginning of the assay, where the only visible alteration is a minor lifting of squamous epithelia (arrowhead). (c) blood capillary; (cc) chloride cell; (e) erythrocyte in capillary; (f) gill filament; gc) goblet (mucous secreting) cell; (is) interlamellar space; (l) gill lamella. (B) Gills of a fish after 14 days of exposure to sediment C (mostly contaminated by organic substances), exhibiting epithelial hyperplasia (hp) and hypertrophy (ht), ultimately leading to fusion ▶

◀ of lamellae (arrow). (hcc) hypertrophied chloride cell. (C) Circulatory disturbances in terminal vessels of lamellae (arrowheads) in a fish exposed to sediment C for 14 days. Erythrocyte accumulation in capillaries and swelling of lamellar tips are evident. (D) Deformed gill lamella (arrow) of a fish exposed to sediment C for 28 days. Desquamation (ds) and hypertrophia (ht) of lamellar epithelial cells are also evident.

Mucous (goblet) cells suffered a very clear regression, in number and size, almost exclusively in C-tested individuals, as revealed by the Alcian blue test (Fig. 3.3.4). This alteration was observed in all C-tested fish collected at $T_{28}$. Degeneration of goblet cells was visible in the entire organ (not limited to occasional foci) and was accompanied by an absence in secreted mucous between lamellae, whereas secreted mucous was observed between the lamellae of gills with normal goblet cells. The largest diameter of normal goblet cells (of $T_0$ individuals) measured 9.3 ± 1.5 µm, whereas that of the atrophied cells (of C-tested fish) was 5.2 ± 1.5 µm. Significant differences were found between the measurements of normal and atrophied cells (Mann-Whitney *U*, $p < 0.01$).

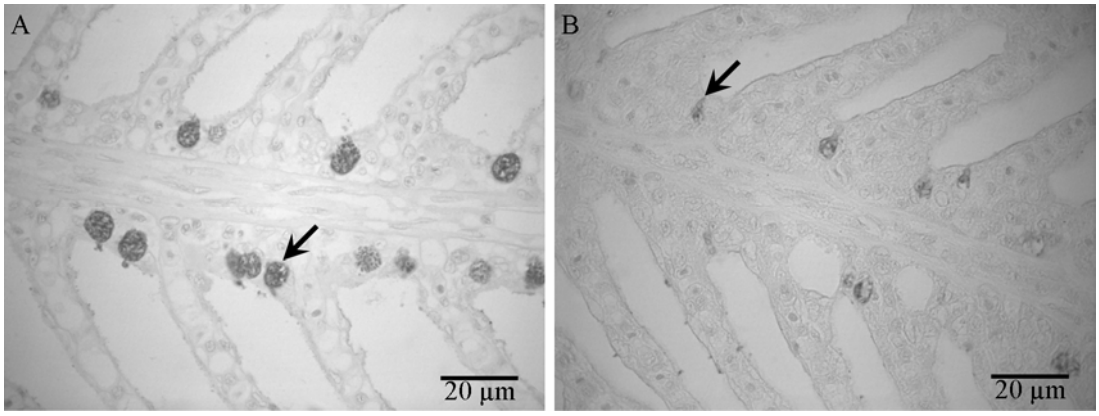


**Fig. 3.3.4.** Mucous (goblet) cells (arrows) in the gills of individuals after 28 days of exposure to tested sediments (AB&NFR). (A) Normal morphology of goblet cells in an individual exposed to the reference sediment (sediment A). (B) Atrophied goblet cells in a fish exposed to essentially organic-contaminated sediments (sediment C).

### *3.5. Histopathological condition weights*

A list of all observed pathologies and respective condition weights is presented in Table 3.3.2. The condition weights were essentially adopted from Bernet et al. (1999), ranging between $w = 1$ and $w = 3$: Necrosis has the maximum value ($w = 3$), followed by granulomatous lesions, hyperplasia, cell atrophy and genetic material-related alterations such as nuclear pleomorphisms (pyknosis and hypertrophy), with $w = 2$. Structural changes in cells and tissues (like hepatocyte vacuolation, squamous cell hypertrophy or lamellar deformation in gills), inflammatory responses (such as blood vessel swelling and presence of melanomacrophages) were given the lowest value ($w = 1$).No specific information exists regarding the biological significance of the presence of eosinophilic bodies in fish cells but some biomedical and pathological research has linked this non-specific alteration to severe lesions such as hepatic neoplasms (Chedid et al., 1999). For this reason, a condition weight $w = 2$,

equal to eosinophilic cellular alteration, was attributed to the presence of eosinophilic bodies. Similarly, little is known about the real significance of chloride cell hypertrophy. Results from exposure to metals indicate that alterations such as hypertrophy and proliferation of these cells have a very important effect on the thickening of epithelia and ion exchange processes, therefore impairing respiration and osmotic balance (Mazon et al., 2002). For this reason, chloride cell hypertrophy in the gills has been given a $w = 2$ value, whereas squamous cell hypertrophy retains the $w = 1$ value proposed by Bernet et al. (1999). Atrophy of gill mucous cells is a first-time described lesion in the present study. Due to the possible severe consequences caused by a deficiency in mucous secretion, which acts as the gill's primary defence barrier to the environment of the animal, it has been attributed a $w = 2$ value.

**Table 3.3.2**. Observed pathologies and their condition weights ($w$).

| **Target organ** | **Reaction pattern** | **Alteration** | ***w*** |
|---|---|---|---|
| *Liver* | Inflammatory response | Profusion and dilation of blood vessels | 1 |
| | | Presence of melanomacrophages | 1 |
| | Regressive | Nuclear pleomorphisms | 2 |
| | | Necrosis | 3 |
| | Progressive | Lipidosis | 1 |
| | | Presence of eosinophilic bodies | 2 |
| | | Eosinophilic hepatocellular alteration | 2 |
| | | Granulomatous lesions | 2 |
| *Gills* | Circulatory disturbances | Lamellar capillary aneurism (telangiectasia) | 1 |
| | Regressive | Epithelial lifting | 1 |
| | | Epithelial desquamation | 1 |
| | | Deformation of lamellae | 1 |
| | | Mucous (goblet) cell degeneration (atrophy) | 2 |
| | Progressive | Hypertrophy of squamous epithelia | 1 |
| | | Lamellar fusion | 1 |
| | | Chloride cell hypertrophy | 2 |
| | | Epithelial hyperplasia | 2 |

### *3.6. Histopathological condition indices*

The two indices were found to be significantly correlated (Spearman $R = 0.60$, $p < 0.01$). In general, both indices revealed highly significant differences between tests and sampling times ($F$ test, $p < 0.01$). The test with sediment C revealed the most significant differences from $T_0$ regarding $I_l$ and $I_g$ at both sampling times, followed by test B, but only for $I_l$. Test A did not cause a significant

increase of either indices in relation to $T_0$ (Fig. 3.3.5). $I_l$ from C-tested individuals collected at $T_{28}$ differed significantly from A- and B-tested fish (Tukey HSD, $p < 0.05$). No significant differences were found between $T_{28}$ and $T_{14}$ $I_l$ and $I_g$, for all tests, although a statistical difference close to the significance threshold was observed between the $I_l$s of C-tested fish collected at $T_{14}$ and $T_{28}$ (Tukey HSD, $p = 0.07$). Regarding gill indices, only C-tested fish showed significant differences from $T_0$ individuals, but no such differences were found in other tests, or between sampling times (Tukey HSD, $p > 0.05$).

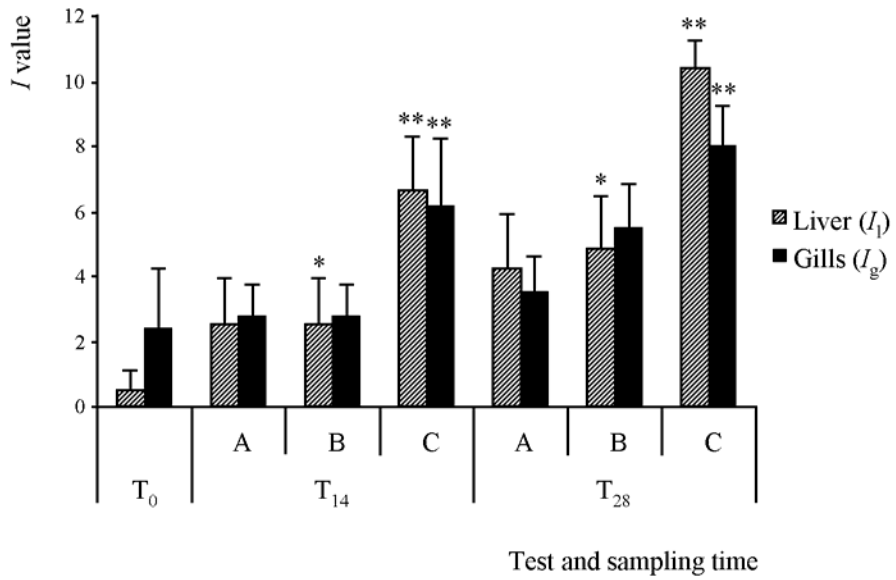


**Fig. 3.3.5.** Mean values of the histopathological condition indices (*I*) obtained from liver and gills of tested individuals. * and * indicate significant differences from $T_0$, $p < 0.05$ and $p < 0.01$, respectively (Tukey's HSD test). Error bars represent 95% confidence intervals.

Cluster analyses derived correlations between lesions (Fig. 3.3.6). Regarding hepatic lesions, three groups of lesions are conspicuous. The first group comprises granulomatous lesions and the presence of melanomacrophages, the second necrosis and eosinophilic bodies, and the third eosinophilic hepatocellular alteration, lipidosis and nuclear pleomorphisms. It is noteworthy, though, that the aforementioned second and third groups can be grouped in a distinct category from the first, according to linkage distances. Blood vessel inflammatory responses are depicted as an independent type of alteration poorly correlated with other lesions. In gills, the strongest correlation was observed between fusion and deformation of lamellae, which, together, are linked to mucous cell atrophy. Aneurysms and epithelial desquamation are also correlated and can be placed in the same cluster of lesions as the previous, forming a distinct group from epithelial hyperplasia and hypertrophy. Chloride cell and epithelial lifting are depicted as the two alterations most detached from other lesions.

PAHs and PCBs were found to be the best correlated sediment contaminants with both condition indices, at both $T_{14}$ and, especially, at $T_{28}$ (Table 3.3.3). Liver indices, however, have in

general better correlations with the surveyed contaminants than gills. Liver indices of individuals collected at $T_{28}$ also depict a significant negative correlation with both growth variables and with the metals Cr, Ni and Zn, a result not observed for gill indices.

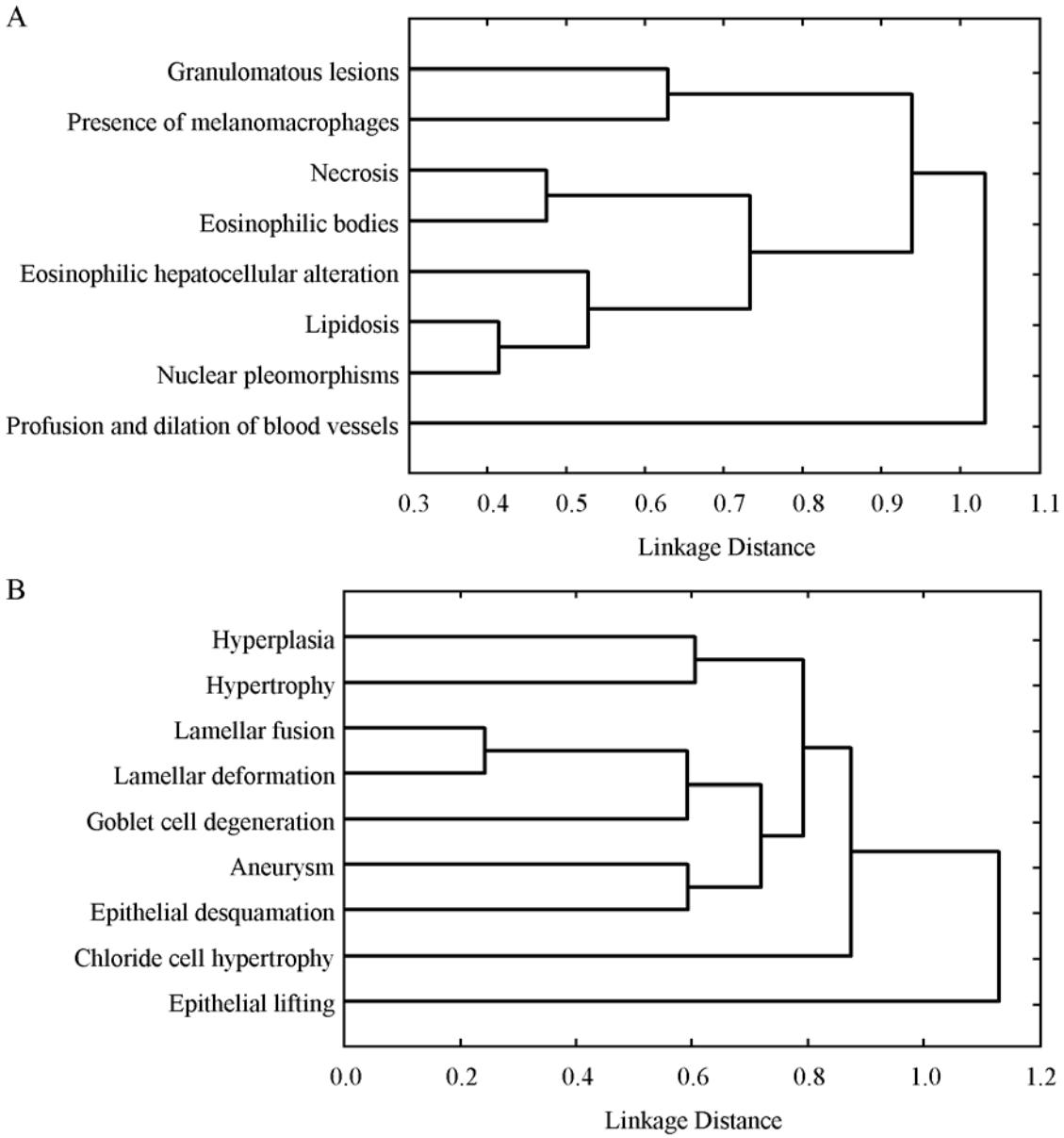


**Fig. 3.3.6.** Joining tree of observed lesions in liver (A) and gills (B) of exposed individuals. Distances were obtained from presence/absence data and estimated as 1-Pearson *r*. Joining is based on unweighted pair-group averages.

## 4. Discussion and conclusions

The present study demonstrated that different profiles of sediment contamination cause distinct patterns of chronic histological lesions in juvenile *S. senegalensis*. These patterns, however, have not shown a linear relationship with cumulative sediment contamination, although exposure to

sediment A (the least contaminated) caused least histopathological lesions and alterations, and exposure to sediment C (mostly contaminated by organic toxicants) caused the most severe lesions in both organs, in accordance with overall mortality. A comparison between the contaminant concentrations of test sediments and some of the most commonly considered sediment quality guidelines (*SQG*s) for coastal areas (MacDonald et al., 1996), namely the threshold effects level (*TEL*) and the probable effects level (*PEL*), suggests that sediment B should have been responsible for the highest toxicity (Table 3.3.4). However, the overall contamination of tested sediments can be considered moderate: *PEL* thresholds are only reached for Cu and Zn and in sediment B only. The severity of lesions observed in test C might be explained by three factors: (1) the highest concentration in sediment C of a few organic compounds, especially some PAHs and PCBs; (2) the synergistic (rather than cumulative) effects of metallic and organic xenobiotics, which may have caused a decrease or a delay in toxicity in B-tested fish; and (3) a higher release of contaminants from sediment C than other sediments during the assay, enhancing toxicant bioavailability.

**Table 3.3.3.** Correlation analyses between condition indices and sediment contaminants plus growth variables.

| Sampling time | | $I_l$ | | $I_g$ | |
|---|---|---|---|---|---|
| | | **Spearman *R*** | ***p*-level** | **Spearman *R*** | ***p*-level** |
| $T_{14}$ | As | 0.60 | 0.00 | 0.42 | 0.03 |
| | Cd | 0.60 | 0.00 | 0.42 | 0.03 |
| | Cr | - | n.s. | - | n.s. |
| | Cu | 0.60 | 0.00 | 0.42 | 0.03 |
| | Ni | - | n.s. | - | n.s. |
| | Pb | 0.60 | 0.00 | 0.42 | 0.03 |
| | Zn | - | n.s. | - | n.s. |
| | tPAH | 0.62 | 0.00 | 0.51 | 0.01 |
| | tPCB | 0.62 | 0.00 | 0.51 | 0.01 |
| | tDDT | 0.60 | 0.00 | 0.42 | 0.03 |
| | $L_s$ | - | n.s. | - | n.s. |
| | $ww_t$ | - | n.s. | - | n.s. |
| $T_{28}$ | As | - | n.s. | 0.40 | 0.04 |
| | Cd | - | n.s. | 0.40 | 0.04 |
| | Cr | -0.63 | 0.00 | - | n.s. |
| | Cu | - | n.s. | 0.40 | 0.04 |
| | Ni | -0.63 | 0.00 | - | n.s. |
| | Pb | - | n.s. | 0.40 | 0.04 |
| | Zn | -0.63 | 0.00 | - | n.s. |
| | tPAH | 0.68 | 0.00 | 0.73 | 0.00 |
| | tPCB | 0.68 | 0.00 | 0.73 | 0.00 |
| | tDDT | - | n.s. | 0.40 | 0.04 |
| | $L_s$ | -0.52 | 0.01 | - | n.s. |
| | $ww_t$ | -0.55 | 0.01 | - | n.s. |

$I_l$ - liver condition indice; $I_g$ - gill condition indice; $L_s$ - fish standard length; $ww_t$ - fish total wet weight; n.s. - non-significant

**Table 3.3.4.** Comparison between the concentrations of surveyed sediment contaminants and available *SQG*s. *TEL* and *PEL* values are given in $mg.kg^{-1}$ sediment dw for metals and $\mu.g^{-1}$ sediment dw for organic substances (from MacDonald et al., 1996).

| Contaminant | | | Site A | Site B | Site C | *TEL*† | *PEL*‡ |
|---|---|---|---|---|---|---|---|
| Metalloid | | As | > *TEL* | > *TEL* | > *TEL* | *7.24* | *41.6* |
| Metal | | Cd | - | - | - | *0.68* | *4.21* |
| | | Cr | - | > *TEL* | - | *52.3* | *160* |
| | | Cu | > *TEL* | > *PEL* | > *TEL* | *18.7* | *108* |
| | | Ni | - | > *TEL* | - | *15.9* | *42.8* |
| | | Pb | - | > *TEL* | > *TEL* | *30.2* | *112* |
| | | Zn | > *TEL* | > *PEL* | - | *124* | *271* |
| PAH | 3-ring | Acenaphthene | - | > *TEL* | - | *6.71* | *88.9* |
| | | Acenaphthylene | - | - | - | *5.87* | *128* |
| | | Anthracene | - | - | - | *46.9* | *245* |
| | | Fluorene | - | - | - | *21.2* | *144* |
| | | Phenanthrene | - | - | - | *86.7* | *544* |
| | 4-ring | Benz[a]anthracene | - | - | > *TEL* | *74.8* | *693* |
| | | Chrysene | - | - | - | *108* | *846* |
| | | Fluoranthene | - | > *TEL* | > *TEL* | *113* | *1494* |
| | | Pyrene | - | - | > *TEL* | *153* | *1398* |
| | 5-ring | Benzo[a]pyrene | - | - | - | *88.8* | *793* |
| | | Benzo[b]fluoranthrene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | | Benzo[e]pyrene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | | Benzo[k]fluoranthrene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | | Dibenzo[a,h]anthracene | - | > *TEL* | > *TEL* | *6.22* | *135* |
| | | Perylene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | 6-ring | Indeno[1,2,3-cd]pyrene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | | Benzo[g,h,i]perylene | *NG* | *NG* | *NG* | *NG* | *NG* |
| | | **tPAH** | **-** | **-** | **-** | ***1,684*** | ***16,770*** |
| PCB | | **tPCB** | **-** | **-** | **-** | ***21.6*** | ***189*** |
| DDT | | *pp*'DDD | - | - | - | *1.22* | *7.81* |
| | | *pp*'DDE | - | - | - | *2.07* | *374* |
| | | *pp*'DDT | - | - | - | *1.19* | *4.77* |
| | | **tDDT** | **-** | > *TEL* | **-** | ***3.89*** | ***51.7*** |

† *TEL* - Threshold effects level: concentration below which contamination effects rarely occur; ‡ *PEL* - Probable effects level: concentration above which contamination effects occur frequently; *NG* - no guideline available; [-] - values below *SQG*s.

The negative correlations found between sediment metals (Cr, Ni, and Zn) and $I_l$ at $T_{28}$ could indicate that, at least at a later stage of exposure, metals may have an antagonistic effect with organic contaminants. The higher damage observed in fish exposed to sediment C, when compared to fish exposed to sediment B, is in accordance with this statement. This interaction between the two classes of contaminants was not observed in the gills since $I_g$ was observed to be positively correlated to sediment contaminants, especially PAHs and PCBs, throughout the experiment. The negative correlation between $I_l$ and body size variables (standard length and total wet weight) indicates that smaller animals may be more susceptible to hepatic chronic lesions as a result from exposure to xenobiotics.

Previous research related hepatocellular alterations such as eosinophilic foci and nuclear abnormalities in fish hepatic tissue to exposure to PAHs and PCBs (Mikaelian et al., 1998; Myers et al., 1998) and linked tissue degeneration, pre-neoplastic and neoplastic diseases to cytochrome activity

and PAH-DNA adducts in flatfish liver (Köhler and Pluta, 1995; Lyons et al., 2004). Detoxification of organic toxicants such as PAHs involves activity of cytochrome P450 (CYP1A) monooxygenase enzymes, to produce the more soluble but highly toxic activated forms (like PAH *o*-quinones) and reactive oxygen species (Flowers-Geary et al., 1996; Burchiel et al., 2007). Metals and metalloids, on the other hand, are known to impair CYP1A induction and activation of PAHs (Vakharia et al., 2001). This synergistic effect between metallic and organic contaminants may contribute to explain the reduced hepatic damage observed in B-tested fish compared to C-tested animals.

Hepatic fatty degeneration observed as lipidosis (intracellular lipid storage in large vacuoles, as opposed to steatosis caused by microvesicular lipid accumulation) was one of the most recurrent alterations found in the livers of fish exposed to sediments B and C. Hepatic lipidosis has been observed in fish exposed to metals (Arellano et al., 1999; Giari et al., 2007), crude oil extracts (Solangi and Overstreet, 1982) and in feral fish from sites contaminated by mixtures of xenobiotics (Greenfield et al., 2008; Triebskorn et al., 2008). Although some authors have discussed that lipid droplets in hepatocytes may store insoluble contaminants or their by-products (Köhler, 1990), this type of alteration has been regarded as a general failure in lipid metabolism as a result of exposure to undifferentiated xenobiotics rather than a specific response (Van Dyk et al., 2007), which is in accordance with the present observations.

The presence of large eosinophilic inclusions in hepatocytes of highly damaged livers is one of the most conspicuous alteration pattern observed. Information is missing regarding the exact causes of this alteration and its consequences to organ function. Eosinophilic bodies in flatfish liver and kidney have already been linked to the exposure to xenobiotics (Camargo and Martinez, 2007; Van Dyk et al., 2007). One of the striking differences, however, between the inclusions observed in the present study and the ones described in the literature is their size. Eosinophilic bodies mentioned in previous studies appear to be much smaller than the ones observed in individuals exposed to sediment C for 28 days. Information on the nature of the substances contained in these inclusions is absent but, considering the affinity of eosin to structural proteins such as actin, it is possible that eosinophilic bodies retain peptide material absorbed from the cytoplasm of degenerating cells. This is supported by data on eosinophilic bodies found in neoplastic areas of human epithelia (Buchner et al., 1976) and liver (Chedid et al., 1999). Considering their correlation to necrosis, eosinophilic bodies may be indicators of severe cirrhosis. Furthermore, their high frequency in the livers of fish exposed to sediment C suggests a link between eosinophilic bodies and exposure to organic contaminants. Altogether, hepatic alterations such as eosinophilic bodies, eosinophilic hepatocellular alteration and lipidosis appear to form a ubiquitous, non-specific group of histopathological alterations within vertebrates, from fish to humans.

The correlation between $I_l$ and $I_g$ may indicate that gills were the major entry organs of contaminants released from the sediments. In fact, no organisms or significant amounts of sediment were found in the guts of tested fish, showing that fish were feeding essentially on pellets. It is thus likely that the digestive system was not primarily involved in the uptake of xenobiotics. Exposure to

metallic and organic contaminants has been linked to acute lesions in gills, like aneurisms and lamellar fusion and, simultaneously, to more severe, chronic hepatic alterations such as lipidosis and neoplastic diseases (Roberts and Oris, 2004; Oliveira Ribeiro et al., 2005). This information is in accordance with the present findings and suggests that gills are more susceptible to the immediate (acute) effects of exposure to waterborne contaminants and livers are subjected to the more prolonged (chronic) effects of accumulated contaminants and their, often more toxic, metabolites. The chronic effects observed in the livers of exposed fish, especially those of B- and C-tested fish indicate prolonged physiological disturbances that led to glycogen depletion and lipid storage, as well as hepatocellular alterations and necrosis. On the other hand, some of the most recurrent gill lesions, namely circulatory disturbances and epithelial hypertrophy and hyperplasia, are known to be reversible (Poleksić and Karan, 1999; Guimarães et al., 2007).

It should be noted that feral animals collected from sites considered to be clean often present a baseline level of non-specific gill lesions. Some authors have suggested that some of these lesions may be originated from parasitosis (Teh et al., 1997; Schwaiger, 2001; Handy et al., 2002). In the present study no indicators of parasites were found in the gills of *S. senegalensis*, which suggests that other environmental parameters may have been the cause of gill lesions observed in fish collected at $T_0$ (such as ammonia levels in the rearing systems). These lesions may be considered as baseline alterations and support the statement that naturally occurring histopathological damage may be an important confounding factor in biomonitoring studies.

One of the most distinctive gill lesions observed was the hypertrophy of chloride cells. Considering the resemblance of hypertrophied cells to empty-like structures, it is unlikely that this alteration was caused by an increase in the metabolic capacities of the cells, which would be reflected in proliferation of mitochondria and endoplasmatic reticuli. It is possible that these alterations cause an imbalance of osmotic regulation by impairing ionic active transport (Mazon et al., 2002). Chloride cell hypertrophy is generally regarded as a response in fish subjected to salinity changes (Karnaky et al., 1976; Foskett et al., 1981) but it has also been found to result from exposure to waterborne pesticides (Fanta et al., 2003). Since water parameters were held constant and kept similar to rearing conditions, chloride cell hypertrophy may have been caused by contaminants released from the sediments.

Another important gill lesion observed in exposed fish was the regression of mucous (goblet) cells, in both number and size. This alteration was observed almost exclusively in C-tested individuals, especially at $T_{28}$, when all surveyed individuals exhibited the pathology. No previous observations of this gill epithelial alteration were found in the literature. The results suggest that atrophy of mucous cells is linked to the characteristics of sediment C, especially its high concentrations of PAHs and PCBs. This alteration may have affected fish health and survival since gill mucous provides vital protection of the delicate structure and epithelia of the gills. The link found between this alteration and structural damage such as lamellar fusion and deformation substantiates this. Also, since mucosubstances act as a primary trap to exogenous substances, it is possible that damage to this barrier increased the exposure to waterborne contaminants. As for chloride cells, the differences found

between the size of normal and atrophied goblet cells indicate that cell measurements may be an important quantitative biomarker.

Sediment collection and handling during the preparation of the assay, combined with the resuspension caused by the scavenging activities of the animals may have enhanced the release of contaminants into the water column. Bioturbation has been found responsible for prolonged bioavailability of the released contaminants (Atkinson et al., 2007). This release may have affected the three tests, contributing to explain some of the damage observed in individuals exposed to sediment A. However, the combination of high anoxia with intermediate FF and TOM contents with sediment resuspension is probably responsible for an increased discharge of contaminants from sediment C (Vale et al., 1998; Caetano et al., 2003; Eggleton and Thomas, 2004).

In conclusion, semi-quantitative indices based on the relative weights of lesions and quantitative data such as the eosinophilic bodies, chloride and goblet cell measurements applied in the present work provide a more biologically realistic and effective approach to analyze histopathological lesions than traditional frequency-based indices. The combination of histopathological indices with several statistical approaches made possible to correlate lesions to sediment contaminants. On the other hand, analysis of frequencies permitted a sensible grouping of histopathological lesions that is in accordance with their biological significance. In addition, laboratory exposures to natural sediments may enhance toxicity by increasing contaminant bioavailability through sediment disturbance; a relevant finding since the assays may have mimicked sediment disturbance events in estuaries such as dredgings, storms and heavy runoffs.

## Acknowledgements

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and RNES (Reserva Natural do Estuário do Sado) for the logistic support and Pedro Pousão (Estação Piloto de Piscicultura, INRB/IPIMAR--Cripsul) for supplying the fish used in the present work.

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## 3.4. A description of chloride cell and kidney tubule alterations in the flatfish *Solea senegalensis* exposed to moderately contaminated sediments from the Sado Estuary (Portugal)[†]


### Abstract

The effects of sediment-bound contaminants on kidney and gill chloride cells were surveyed in juvenile *Solea senegalensis* exposed to fresh sediments collected from three distinct sites of the Sado Estuary (Portugal) in a 28-day laboratorial assay. Sediments were analyzed for metallic contaminants, polycyclic aromatic hydrocarbons and organochlorines as well as for total organic matter, redox potential and fine fraction. The potential for causing adverse biological effects of each surveyed sediment was assessed by comparison of contaminant levels to available guidelines for coastal sediments, namely the Threshold Effects Level (*TEL*) and the Probable Effects Level (*PEL*). The Sediment Quality Guideline Quotient indices (*SQG-Q*) were calculated to compare the overall contamination levels of the three stations. A qualitative approach was employed to analyze the histo/cytopathological traits in gill chloride cells and body kidney of fish exposed to each tested sediment for 0, 14 and 28 days. The results showed that sediment contamination can be considered low to moderate and that the least contaminated sediment (from a reference site, with the lowest *SQG--Q*) caused lesser changes in the surveyed organs. However, the most contaminated sediment (by both metallic and organic xenobiotics, with highest *SQG-Q*) was neither responsible for the highest mortality nor for the most pronounced lesions. Exposure to the sediment presenting an intermediate *SQG-Q*, essentially contaminated by organic compounds, caused the highest mortality (48%) and the most severe damage to kidneys, up to full renal necrosis. Chloride cell alterations were similar in fish exposed to the two most contaminated sediments and consisted of a pronounced cellular hypertrophy, likely involving fluid retention and loss of mitochondria. It can be concluded that sediment contamination considered to be low or moderate may be responsible for severe injury to cells and parenchyma involved in the maintenance of osmotic balance, contributing for the high mortality levels observed. The results suggest that sediment-bound organic contaminants such as PAHs (polycyclic aromatic hydrocarbons) and PCBs (polychlorinated biphenyls) may be very toxic to the analyzed organs, especially the kidney, even when present in low risk concentrations.


[†] Costa et al. (2010) *J. Sea Res.* **64**:465-472 (doi:10.1016/j.seares.2010.01.005).

**Key-words**

Senegalese Sole; Chloride Cell Hypertrophy; Nephrotoxicity; Histopathology; Sediment Contaminants; Estuarine Environment

## 1. Introduction

Osmotic regulation is a vital function in marine fish due to the hypertonic nature of their environment. Estuarine fish, on the other hand, require some plasticity of the mechanisms that maintain their internal osmotic balance due to the salinity fluctuations of their environment. In either case, the failure of osmotic balance structures implicates severe stress to fish and overall loss-of-fitness to their habitat. Kidneys and chloride cells are the most important structures for osmotic balance in fish, although with different functions: whereas the gill chloride cells in fish have long been described to maintain internal osmotic balance by actively excreting or uptaking ions (Keys and Wilmer, 1932); the secretory component of the renal system such as the body (trunk) kidney of fish is known to act as a primary system for the elimination of organic xenobiotic metabolites as part of ion excreting processes (see Pritchard and Miller, 1997 for a review). Toxic metals, however, may only negligibly be excreted by the kidneys but are known to severely impair renal ion excretion functions (Leffler et al., 2000; Chowdhury and Wood, 2007). For these reasons, many authors have focused on the effects of environmental contaminants on the function and morphology of these structures (e.g. Triebskorn et al., 2002, 2004; Giari et al., 2007). Nevertheless, specific information regarding lesions and alterations to osmotic regulating structures is scarce, especially regarding the effects of complex mixtures of contaminants as in natural sediments and the differential toxicity of the various classes of contaminants.

Histopathological biomarkers have long been surveyed in benthic fish with the purpose of monitoring estuarine sediment contamination. Histopathology is frequently considered a more realistic tool than biochemical approaches to assess toxicity for directly reflecting fish health, thus are more effectively being extrapolated to community-level effects of contamination (Stentiford et al., 2003; Au, 2004). Despite the growing amount of research on fish cyto/histopathology, this approach still suffers from many constraints, ranging from terminology discrepancies to the difficulties in establishing cause-effect relationships between environmental parameters and pathological traits. Conversely, while liver and overall gill structure have been widely surveyed, specific alterations of chloride cells and body kidney tubules still need further research.

The Senegalese sole, *Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae), is a benthic teleost of important value for fisheries and aquaculture in Southern Europe. The species inhabits soft bottoms of coastal areas, especially estuaries, which function as breeding and nursing grounds, where it feeds on small invertebrates (Cabral and Costa, 1999; Cabral, 2000; Sá et al., 2003).

Combined with its relative abundance, these characteristics contribute to the species' projected value as a sentinel for environmental contamination (Jiménez-Tenorio et al., 2007).

The Sado Estuary is a large confined coastal area where the effort to preserve environmental quality and sustain human development has dictated an attempt to monitor environmental contamination and its effects on organisms. The estuary is subjected to different sources of contamination: urban from the city of Setúbal, industrial from the city's heavy-industry belt (one of the largest in Portugal) and agricultural from the grounds upstream. The estuary is also an important harbour area, with several shipyards and port facilities, for such reason being subjected to regular dredging. The estuary is also very important for local fisheries, aquaculture and tourism, each representing an important fraction of the local economy. A large part of the estuary is classified as a natural reserve and the only Portuguese underwater reserve on the mainland territory is located just off the estuary. Current environmental monitoring procedures to assess sediment contamination are being performed on three representative stations of the Sado Estuary, selected according to previous information (Caeiro et al., 2005; Neuparth et al., 2005; Costa et al., 2008). These stations (Fig. 3.4.1) have different levels of contamination and different sediment and hydrodynamical characteristics: site A is the station farthest from contamination sources and the site with highest hydrodynamics and lower water residence time. Sites B and C (located off the city of Setúbal and the industrial belt, respectively) are the most contaminated, although with distinct patterns of contamination by metallic and organic xenobiotics.

The present work aimed at the identification and description of histological lesions and alterations in kidneys and gill chloride cells in juvenile *S. senegalensis* through a wide set of histological procedures and to relate sediment contamination to toxicity, using a sediment quality indices approach to determine the potential impact on organisms.

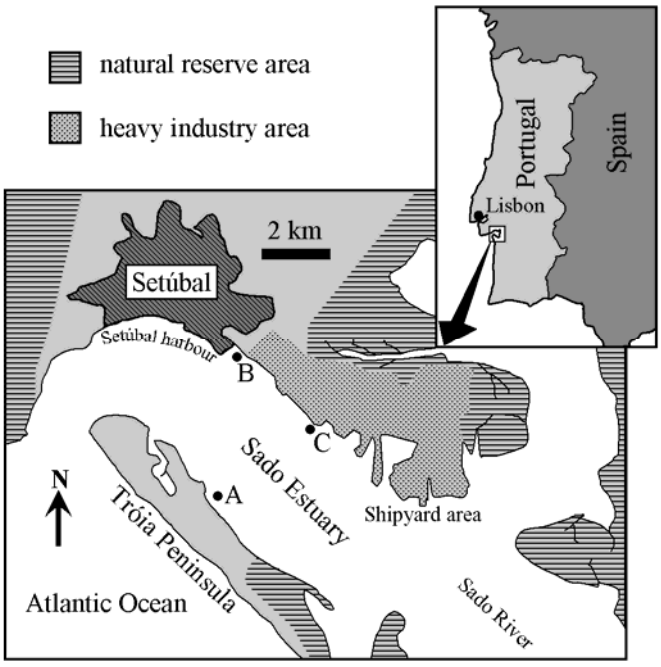


**Fig. 3.4.1.** Map of the Sado Estuary showing the selected study sites A, B and C (•).

## 2. Methods and materials

### *2.1. Experimental procedure*

The sediments from the selected sites, A, B and C (Fig. 3.4.1), were collected with a grab on November 2006. Sediments were subdivided and frozen for analyses (detailed below) while fresh portions were used to prepare the assays after having been stored at 4 °C for a period not extending 5 days. Sediments were homogenized after collection with a spatula to ensure similarity between the samples to be used in the assays and those analyzed for contaminants. The 28-day bioassays were performed with two replicates per treatment, in a closed system tank arrangement with constant aeration and water recirculation (regulated to prevent any hydrodynamic-driven sediment resuspension). Two litres of sediment was allocated in 15 L-capacity polyvinyl tanks with blunt edges (providing approximately a sediment surface of 525 $cm^2$ and a depth of 3-4 cm) and was added 12 L of clean seawater. Sediments were let to settle for 48 h before the beginning of the assay. Twenty-four randomly selected hatchery-brood and laboratory-reared juvenile *S. senegalensis*, all from the same cohort (69 ± 6 mm standard length), were placed in each tank. A partial weekly water change was made (25% of total water volume) to maintain constant the water parameters: pH 7.9 ± 0.2, salinity = 33 ± 1 $g.L^{-1}$, total ammonia = 3 ± 1 $mg.L^{-1}$. Water temperature was set at 18 ± 1 °C, $O_2$ saturation ranged between 40 and 45% and the photoperiod was set at 12:12 h light:dark. The water parameters were monitored weekly to ensure constancy and were found to be equal to the rearing systems. Animals were fed daily with commercial pelleted food for aquaculture fish (Aquasoja M2 grade, from Sorgal, Portugal). For simplification purposes, exposure to the three sediments will hereon be referred to as tests A, B and C. Twelve fish per test (six per replica) were collected for analysis at each sampling time, i.e., at days 14 ($T_{14}$) and 28 ($T_{28}$). Fish collected at day 0 ($T_0$) consisted of twelve animals from the rearing tanks.

### *2.2. Sediment characterization*

Sediment redox potential (Eh) was measured immediately after collection and homogenization, using an Orion model 20A apparatus equipped with a H3131 Ag/AgCl reference electrode. In addition, sediments were analyzed for organic matter (OM) and fine fraction (particle size < 63 μm) contents by organic carbon loss-on-ignition (LOI) at 500 ± 50 °C and hydraulic sieving, respectively. Both results are expressed as percentage relatively to sediment dry weight. Sediment contaminants (metallic, polycyclic aromatic hydrocarbons and organochlorines) were selected according to previous findings for the same proximate areas, where levels of concern have been found (see Caeiro et al., 2005; Neuparth et al., 2005; Costa et al., 2008). Metallic sediment contaminants were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series spectrometer after total digestion of sediment samples with a mixture of acids

(HF, HCl, and $HN0_3$) in closed Teflon vials, according to the procedure described by Caetano et al. (2007). The procedure was validated by analysis of the reference sediments MESS-2 (NRC, Canada), PACS-2 (National Research Council, Canada) and MAG-1 (USGS, USA), having found the obtained concentrations within the certified ranges. Polycyclic aromatic hydrocarbons (PAHs) were determined using a GCQ Trace Finnigan gas chromatography-mass spectrometry (GC-MS) apparatus in selected ion mode (SIM) after Soxhlet-extraction with an acetone-hexane (1:1) mixture, as described by Martins et al. (2008). Polychlorinated biphenyl (PCBs) congeners and the pesticide residues *pp*'DDD (1,1-dichloro-2,2-bis[*p*-chlorophenyl]ethane), *pp*'DDE (l,l-dichloro-2,2-bis[*p*-chlorophenyl]ethylene) and *pp*'DDT (l,l,l-trichloro-2,2-bis[*p*-chlorophenyl]ethane) were analyzed by GC-MS using a Hewlett--Packard 6890 gas chromatograph with an electron capture detector (ECD) after Soxhlet extraction with *n*-hexane (Ferreira et al., 2003). Quality control for organic contaminant determination was obtained from an analysis of the reference sediment SRM 1941b (NIST, USA) and the measured values were found within the certified ranges.

The impact potential for observing adverse biological effects of the tested sediments was evaluated by calculating the *PEL* quotient (*PEL-Q*) based on the published guideline values for coastal waters, namely the Threshold Effects Level (*TEL*) and the Probable Effects Level (*PEL*), according to MacDonald et al. (1996). These guidelines were originally developed for coastal waters and have been largely used in estuarine sediment ecological risk assessment studies. The *PEL-Q* indices were calculated for each contaminant according to the formula described by Long and MacDonald (1998):

$$PEL - Q_i = \frac{C_i}{PEL} \qquad [1]$$

Where *PEL* is the guideline value for the contaminant *i* and $C_i$ the measured concentration of the contaminant in the surveyed sediment. The Sediment Quality Guideline Quotient indice (*SQG-Q*), developed to compare sites impacted by mixtures of contaminants, was calculated for each sediment according to Long and MacDonald (1998) as:

$$SQG - Q = \frac{\sum_{i=1}^{n} PELQ_i}{n} \qquad [2]$$

Where $PEL\text{-}Q_i$ is the indice deriving from [1] for the contaminant *i* and n the number of contaminants under analysis. Stations were scored according to their overall potential of observing adverse biological effects, as proposed by MacDonald et al. (2004): *SQG-Q* < 0.1 - unimpacted; 0.1 < *SQG-Q* < 1 - moderately impacted; *SQG-Q* < 1 - highly impacted.

*2.3. Histological analyses*

Animals were anaesthetized on ice and euthanized by cervical sectioning. Organ samples (first and second gills arches from the eyed side and body kidney) were immediately excised and fixed in Bouin-Hollande's solution (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added to saturation) for 48 h. Samples were then washed for 24 h in distilled water (kidney samples) or in a 6% v/v formic acid solution to promote decalcification (gills). Samples were afterwards dehydrated in a progressive series of ethanol and embedded in paraffin (xylene was used for intermediate impregnation). The procedure from fixation to embedding was adapted from Martoja and Martoja (1967). For both organs, 2-3 μm thick sections were obtained. Slides were prepared in duplicate for each organ and staining procedure, with 6-8 sequential sections per slide. Kidney and gill were stained with haematoxylin and eosin (H&E) for structural analyses as described by Martoja and Martoja (1967). Gill sections were additionally stained through the Alcian blue histochemical method using nuclear fast red as counterstain (AB&NFR) for identification of goblet (mucous secreting) cells (Kiernan, 2008) and with the acridine orange fluorochrome stain (AO) for nucleic acids to identify mitochondria in chloride cells (Costa and Costa, 2008). Slides were cleared with xylene and mounted in DPX resinous media (BDH). A DMLB model microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps and an I3 filter was used for analyses. All equipments were supplied by Leica Microsystems. Identification of normal and pathological features on gills and kidneys was primarily based on Hibiya (1982) and Arellano et al. (1999, 2004).

*2.4. Statistical analyses*

Cell measurements and counts were analyzed by the nonparametric Kruskall-Wallis ANOVA by ranks $H$ ($\alpha = 0.05$). Statistics were computed with the software Statistica (Statsoft Inc.), according to Zar (1998).

## 3. Results

Overall mortality at the end of the assays was much differentiated between tests: 2% as result of exposure to sediment A, 13% for test B and 48% for C.

*3.1. Sediment characterization*

The tested sediments revealed distinct characteristics and patterns of contamination (Table 3.4.1). Sediment A, collected from the reference site, was the least contaminated and the least anoxic. Sediment C was essentially contaminated by organic compounds and was found to have the lowest

redox potential, whereas sediment B was contaminated by metallic and organic toxicants and was the sediment with highest OM and FF percentages. Inference on *SQG-Q* indices (equation 2) indicate that site A is unimpacted while sites B and C are moderately impacted, with the sediments from site B presenting the highest potential ecological hazard, according to the classification proposed by MacDonald et al. (2004). Contamination of sediments B and C can, however, be considered low to moderate, since *PEL* thresholds are only reached for the elements copper (Cu) and zinc (Zn) in sediment B.

*3.2. Gill and chloride cell histopathology*

Fish collected at $T_0$ essentially exhibited normal gill morphology of filaments and lamellae (Fig. 3.4.2A). No considerable amounts of sediment particles were found in the gills and fish did not exhibit evidence for gill parasites. Normal gill epithelium on filament and lamella is typically formed by a single layer of cells. Chloride cells stained with H&E have a densely-stained granulous cytoplasm. Discrimination of chloride cells from goblet (mucous secreting) and regular epithelial cells could be aided by the observation of the crypt opening to the exterior and by the vesicular aspect of goblet cells' cytoplasm, which differentially retains the haematoxylin pigment according to the mucosubstances' pH. Staining with the nuclei-acid specific fluorochrome allows good discrimination of chloride cells due to the fluorescence of mitochondrial DNA (Fig. 3.4.2B). Chloride cells are mostly located in the interlamellar epithelium of the filament (≈ 2 ± 1 cells between lamellae) but smaller, flattened; chloride cells can occasionally be observed between lamellar pavement cells. Structural lesions in gills of exposed fish were in general moderate and more frequent in individuals exposed to sediment B and moreover to sediment C. Gill damage was observed, however, to increase from $T_0$ to $T_{28}$ for all tests. Some of the most recurrent alterations consisted of moderate interlamellar hyperplasia (proliferation) and hypertrophy of lamellar pavement cells (although no rod-shaped filaments were observed). In fish exposed to sediments B and C, however, hypertrophy of chloride cells was a frequent alteration (Fig. 3.4.3). Chloride cell hypertrophy was uncommon on A-tested fish or restricted to small localized foci, whereas in B and C-tested individuals this alteration was typically observed throughout the gill arches (one or two hypertrophied cell were commonly observed in basal interlamellar epithelia, and could also occasionally be found in lamellar epithelia). Hypertrophied chloride cells appeared as empty, vacuole-like structures (loosing their typical granular cytoplasm) that could be positively discriminated from other epithelial cells by differential staining (Fig. 3.4.4A--C). Nuclei and remaining cytoplasmatic structures were observed compressed against the cell's membrane, depending on the section under observation. These cells were found to be ellipsoidal in shape. The average largest axle of the hypertrophied chloride cells measured 17 ± 2 μm and no statistical differences were found between tests and sampling times (Kruskall-Wallis $H$ = 9.57, $p$ = 0.09). Due to cell enlargement, the surrounding tissue was often compressed. No significant changes were observed in the total number of chloride cells (normal/hypertrophied) in the

interlamellar basal epithelium between all tests and sampling times (Kruskall-Wallis $H$ = 1.79, $p$ = 0.77).

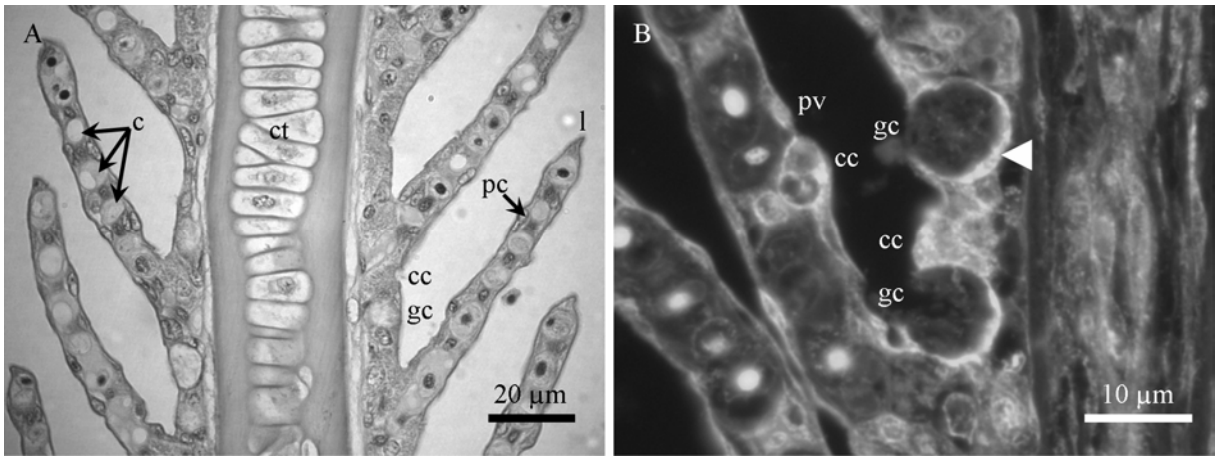


**Fig. 3.4.2.** Normal morphology of gills of a fish collected at the beginning of the assay ($T_0$). A) Bright-field micrograph (H&E). c) Lamellar capillaries; cc) chloride cell where the crypt opening to the exterior is clearly visible; ct) gill filament supporting cartilage; gc) goblet (mucous secreting) cell; l) gill lamella; pc) pillar cell. B) Epifluorescence micrograph (AO) of a normal gill, exhibiting chloride cells in lamella and filament epithelia (cc). Chloride cells are strongly fluorescent and have a granular cytoplasm due to their high concentration of mitochondria. Goblet cells (gc) also possess a highly fluorescent cytoplasm typically compressed against the cell's basal surface (arrowhead) but most of the cell's intraplasmatic space is occupied by mucous vesicles that do not retain the dye. Non-secreting epithelial cells such as pavement cells (pv) do not exhibit a densely fluorescent cytoplasm.

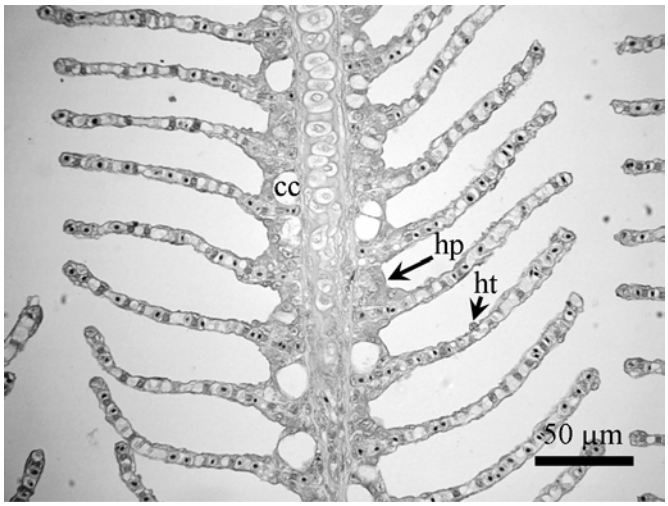


**Fig. 3.4.3.** Overview of the gills of a fish exposed to sediment C (mostly contaminated by organic compounds) for 28 days (H&E), exhibiting many hypertrophied chloride cells (cc). Structural damage to the gills is low but foci of moderate pavement cell hyperplasia (hp) can be observed in the interlamellar spaces, as well as hypertrophied pavement cells (ht).

**Table 3.4.1.** General characterization and contaminant ranges of tested sediments. *PEL* and *TEL* guidelines were obtained from MacDonald et al. (1996).

| | | | | | Site | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | **A** | | **B** | | **C** |
| | | Organic matter (%) | | | 3 | | 12 | | 8 |
| | | Fine fraction (%) | | | 37 | | 98 | | 77 |
| | | Redox potential (mV) | | | -233 | | -290 | | -316 |
| Contaminant class | | | *TEL*[a] | *PEL*[b] | | *PEL-Q*[c] | | *PEL-Q*[c] | | *PEL-Q*[c] |
| **Metallic (µg.g$^{-1}$ sediment dw)** | | | | | | | | | | |
| As | | | 7.24 | 41.6 | 7.25 ± 0.15* | 0.17 | 27.43 ± 0.55* | 0.66 | 12.38 ± 0.25* | 0.30 |
| Cd | | | 0.68 | 4.21 | 0.04 ± 0.00 | 0.01 | 0.22 ± 0.00 | 0.05 | 0.15 ± 0.00 | 0.04 |
| Cr | | | 52.3 | 160 | 24.20 ± 0.48 | 0.15 | 76.33 ± 1.53* | 0.48 | 21.85 ± 0.44 | 0.14 |
| Cu | | | 18.7 | 108 | 22.57 ± 0.45* | 0.21 | 167.32 ± 3.35** | 1.55 | 41.18 ± 0.82* | 0.38 |
| Ni | | | 15.9 | 42.8 | 12.97 ± 0.26 | 0.30 | 33.67 ± 0.67* | 0.79 | 9.03 ± 0.18 | 0.21 |
| Pb | | | 30.2 | 112 | 23.70 ± 0.47 | 0.21 | 66.49 ± 1.33* | 0.59 | 45.17 ± 0.90* | 0.40 |
| Zn | | | 124 | 271 | 147.48 ± 2.95* | 0.54 | 312.23 ± 6.24** | 1.15 | 87.75 ± 1.76 | 0.32 |
| **Organic (ng.g$^{-1}$ sediment dw)** | | | | | | | | | | |
| | 3 - ring | Acenaphthene | 6.71 | 88.9 | 1.41 ± 0.24 | 0.02 | 9.42 ± 1.60* | 0.11 | 4.19 ± 0.71 | 0.05 |
| | | Acenaphthylene | 5.87 | 128 | 0.24 ± 0.04 | 0.00 | 1.83 ± 0.31 | 0.01 | 1.95 ± 0.33 | 0.02 |
| | | Anthracene | 46.9 | 245 | 1.03 ± 0.17 | 0.00 | 10.60 ± 1. | 0.04 | 15.34 ± 2.61 | 0.06 |
| | | Fluorene | 21.2 | 144 | 1.32 ± 0.22 | 0.01 | 8.70 ± 1.48 | 0.06 | 8.03 ± 1.37 | 0.06 |
| | | Phenanthrene | 86.7 | 544 | 7.96 ± 1.35 | 0.01 | 50.77 ± 8.63 | 0.09 | 54.09 ± 9.20 | 0.10 |
| PAHs | 4 - ring | Benz[a]anthracene | 74.8 | 693 | 4.53 ± 0.77 | 0.01 | 64.60 ± 10.98 | 0.09 | 86.52 ± 14.71* | 0.12 |
| | | Chrysene | 108 | 846 | 2.20 ± 0.37 | 0.00 | 28.31 ± 4.81 | 0.03 | 37.19 ± 6.32 | 0.04 |
| | | Fluoranthene | 113 | 1494 | 18.05 ± 3.07 | 0.01 | 170.80 ± 29.04* | 0.11 | 184.30 ± 31.30* | 0.12 |
| | | Pyrene | 153 | 1398 | 14.66 ± 2.49 | 0.01 | 131.74 ± 22.40 | 0.09 | 171.39 ± 29.14* | 0.12 |
| | 5 - ring | Benzo[a]pyrene | 88.8 | 793 | 7.56 ± 1.28 | 0.01 | 69.81 ± 11.87 | 0.09 | 85.88 ± 14.60 | 0.11 |
| | | Dibenzo[a,h]anthracene | 6.22 | 135 | 0.74 ± 0.13 | 0.01 | 7.45 ± 1.27* | 0.06 | 6.99 ± 1.19* | 0.05 |
| PCBs | | ΣPCB | 21.6 | 189 | 1.87 ± 0.32 | 0.01 | 5.64 ± 0.96 | 0.03 | 15.34 ± 2.61 | 0.08 |
| | | *pp*'DDD | 1.22 | 7.81 | 0.10 ± 0.02 | 0.01 | 0.28 ± 0.05 | 0.04 | 0.60 ± 0.10 | 0.08 |
| DDTs | | *pp*'DDE | 2.07 | 374 | 0.05 ± 0.01 | 0.00 | 0.27 ± 0.05 | 0.00 | 0.65 ± 0.11 | 0.00 |
| | | *pp*'DDT | 1.19 | 4.77 | 0.70 ± 0.12 | 0.15 | 4.39 ± 0.75* | 0.92 | 1.18 ± 0.20 | 0.25 |
| | | ***SQG-Q***[d] | | | | **0.08** | | **0.32** | | **0.14** |

[a] Threshold Effects Level - concentration below which contamination effects rarely occur; [b] Probable Effects Level - concentration above which contamination effects occur frequently; [c] *PEL* quotient (equation 1); [d] Sediment Quality Guideline quotient (equation 2); * Concentrations above *TEL*; ** Concentration above *PEL*

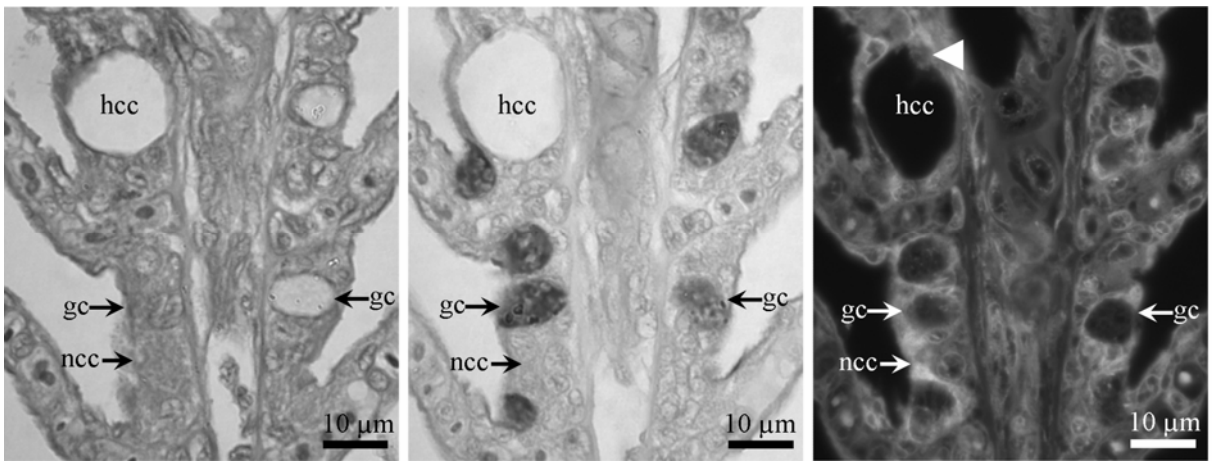


**Fig. 3.4.4.** Sequential sections of the gills of a fish exposed to sediment B (contaminated by metallic and organic xenobiotics) for 14 days where hypertrophied (hcc) and normal (ncc) chloride cells can be observed, among many goblet cells. Sections are stained by different techniques: A) H&E; B) AB&NFR for mucosubstances; C) AO for DNA (epifluorescence micrograph). Normal chloride cells retain the typical granulous, dense, cytoplasm, whereas hypertrophied cells appear as empty-like, unstained structures, indicating probable fluid retention. Goblet cells appear weakly or strongly stained by haematoxylin depending on mucous pH (basic or acid, respectively), but are always stained by the Alcian blue reaction and are thus well distinguishable from other epithelial cells. Hypertrophied chloride cells do not exhibit the strong AO fluorescence of mitochondria-rich cytoplasm in normal cells but the nucleus (arrowhead) is retained.

*3.3. Body kidney histopathology*

Fish exposed to sediments B and C sustained strong renal damage when compared to A-tested individuals, which depicted normal kidneys, similar to those observed in $T_0$-collected fish (Fig. 3.4.5A). Individuals collected at $T_0$ and those exposed to sediment A for 14 and 28 days exhibited normal kidney tubules (renal parenchyma) and a normal hematopoietic interstitial (intertubular) tissue surrounding the tubules (mostly containing erythrocytes and blast cells). As typical in saltwater fish, glomeruli were found to be rare or even absent and reduced in size when present. A prominent regression of tubules was observed in fish exposed to sediments B and C at the assays' midterm ($T_{14}$). Cloudy swelling (also termed albuminous degeneration) became an evident alteration of tubular epithelial cells, resulting in cell hypertrophy (and loss of shape) with a granular cytoplasm. Altered cells did not appear to have nuclear alterations. With respect to tubular structure, a very evident regression was observed, typically associated to degenerating or necrotic cloudy-swollen cells. This lesion implied the loss of tubule shape, reduction of lumen diameter and, frequently, tubule disappearance. Necrosis appears to be preceded by cloudy swelling and followed by tubule disorganization and disappearance. However, whereas in the kidneys of B-tested fish collected at this sampling time some intact tubules could be found (Fig. 3.4.5B), in fish exposed to sediment C the regression of tubules was more severe (Fig. 3.4.5C). The most severe damage was observed in fish exposed to sediment C for 28 days, with some individuals exhibiting entirely necrotic sections, affecting both tubular and hematopoietic tissue. Many melanomacrophages and haemosiderin deposits

(appearing as reddish granules) were observed on necrotic tissue. Kidneys of B-tested fish did not exhibit significant changes in kidney damage from $T_{14}$ to $T_{28}$. No clear evidence for mitosing cells was in the renal parenchyma of fish exposed to sediments B and C, at both sampling times. Due to the reduced number of Malpighian corpuscles, glomerular lesions could not be scored.

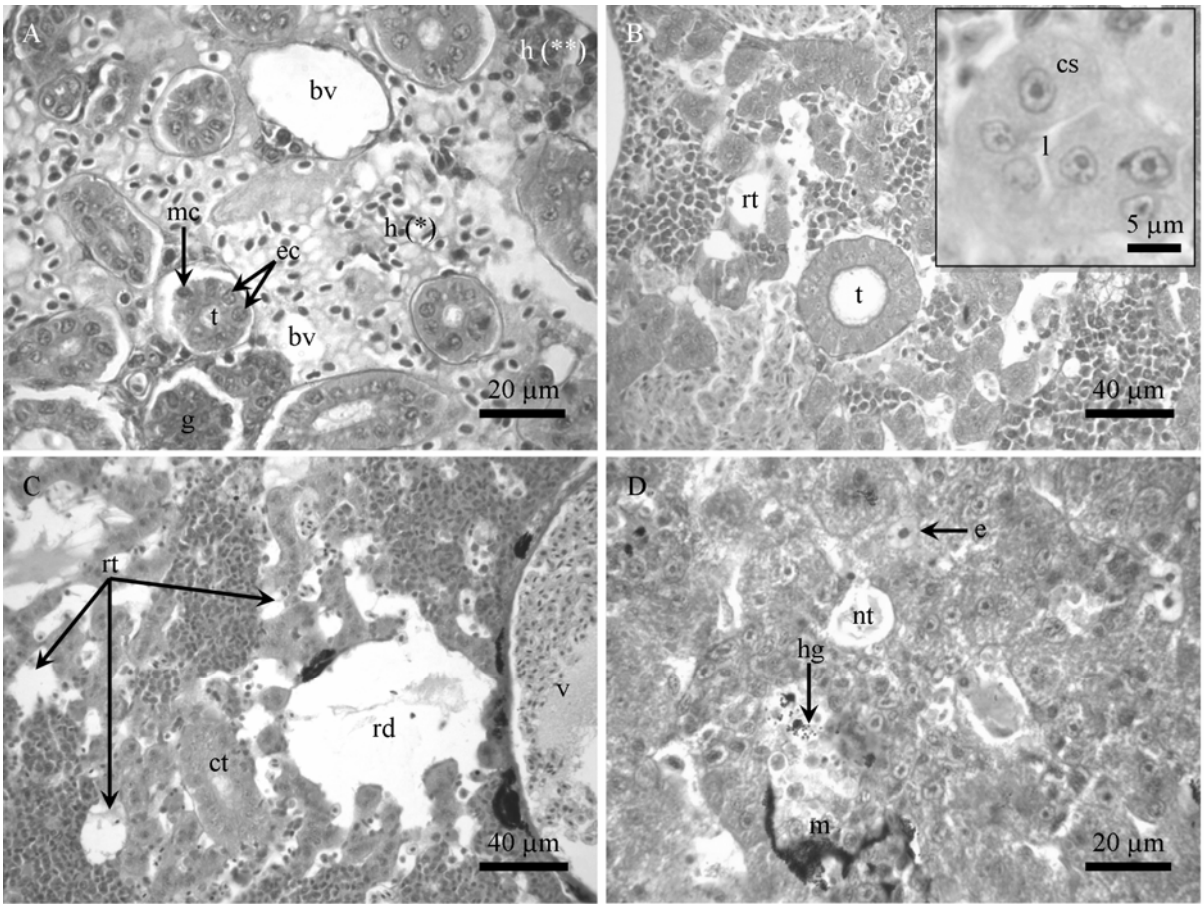


**Fig. 3.4.5.** Body kidney (proximal convoluted segment) morphology of exposed individuals (H&E). A) Normal kidney of an individual collected at $T_0$. bv) blood vessels; g) glomerulus inside a Malpighian corpuscle; h) haematopoietic tissue (with *erythrocytes and **blast cells); t) renal tubule lined by normal cuboidal epithelial cells (ec), internally lined by a microvili layer. Mitosing cells (mc) could frequently be observed, indicating parenchyma regeneration. B) Overview of the kidney of a fish exposed to sediment B (contaminated with metallic and organic substances) for 14 days, exhibiting normal (t) among many regressing (rt) tubules formed by cloudy-swollen and necrotic cells. Side-panel: detail of a tubule lined by cloudy-swollen epithelial cells (cs), from the same sample, an alteration that typically causes a pronounced reduction of the lumen (l) diameter. C) Overview of the renal parenchyma of a fish after 14 days of exposure to sediment C (essentially contaminated by organic toxicants), exhibiting tubules completely lined by cloudy-swollen epithelial cells (ct) and regressing tubules (rt). A regressing urinary duct is also evident (rd). v) renal vein with erythrocytes inside. D) Detail of the heavily-damaged kidney of a fish exposed to sediment C for 28 days, exhibiting acute necrosis of parenchymal and interstitial tissue with melanomacrophages (m) and haemosiderin granules (hg). The few persisting tubules (nt) are clearly lined by necrotic epithelial cells. The haematopoietic (intertubular) tissue is also suffering from advanced necrosis, with few, sparse, intact erythrocytes (e) or blast cells.

## 4. Discussion

The present work demonstrated that exposure to sediments that might be considered low to moderately contaminated may cause severe damage to the kidneys and gill chloride cells in juvenile Senegalese soles. The severity of lesions and overall mortality were not, however, directly related to the cumulative sediment contamination since exposure to sediment C, to which was obtained an intermediate *SQG-Q* contamination indice (valuing 0.14, whereas sediments A and B *SQG-Q*s were 0.08 and 0.32, respectively), caused greater mortality and more pronounced kidney alterations. Still, exposure to both most contaminated sediments (B and C) caused high damage to kidneys and gill chloride cells, probably compromising the animals' capability to regulate their internal osmotic balance. The similarity between chloride cell alterations in fish exposed to both sediments and the higher severity of renal lesions observed in fish exposed to sediments essentially contaminated by organic compounds (namely, sediment C) may indicate that organic xenobiotics, especially PAHs and PCBs were the primary xenobiotics accountable for toxicity, even though the action of well represented metals such as Cu and Zn should not be excluded.

The observed chloride cell alterations imply a change in the cellular structure by fluid retention (causing cell enlargement) and loss of mitochondria and other nucleic acid-bearing cytoplasmatic structures like the rough endoplasmatic reticulum. These findings are in accordance to the chloride cell dystrophies observed by Arellano et al. (1999) in *S. senegalensis exposed* to waterborne copper. The presence of intact nuclei and plasmatic membranes indicate that hypertrophy of chloride cells is not directly related to necrosis. This alteration is, nevertheless, likely to impair active transport of ions due to a reduction in the number of mitochondria and consequently in cellular energy production. Chloride cell hypertrophy and hyperplasia in fish gills have been found to be an adaptation to hypertonic media (Karnaky et al., 1976b; Foskett et al., 1981). These changes imply an increase in cell activity and microstructure density (Karnaky et al., 1976a; Foskett et al., 1981). However, the alterations observed in the present work indicate loss of subcellular structures and fluid retention in chloride cells of fish exposed to contaminated sediments. Due to the constancy of the assays' water parameters, it can be reasoned that the observed hypertrophy is putatively caused by xenobiotics released from the sediments and not by osmotic-driven chloride cell hypertrophy. The hypertrophied cells observed in the gills of fish exposed to sediments B and C, rather than being a contaminant-triggered overresponse to environment salinity should actually result from atrophy/regression of cellular constituents. In fact, chloride cell hypertrophy and impairment of gill active transporter enzymes have already been found to be linked to exposure to different classes of xenobiotics, from metal to pesticides (Arellano et al., 1999; Fanta et al., 2003; Monette et al., 2008), which substantiates the present findings and indicates that this alteration is an unspecific effect of contamination. It is noteworthy that no traces of sediment particles or organisms were found in the digestive tracts of exposed fish, what may indicate that fish were feeding essentially on pellets. It can therefore be argued that gill epithelium was the principal entry point of contamination.

Many of chronic histopathological traits described by toxicological studies in fish and other vertebrates, such as tubule cell vacuolation and peptide hyaline inclusions (e.g. Triebskorn et al., 2004; Camargo and Martinez, 2007; McCoy et al., 2008), were not observed in the present study. Instead, the major alterations observed, such as cloudy swelling, necrosis and tubule regression, combined with the absence of tubular regeneration, should indicate severe, acute damage to the body kidney of fish exposed to the most contaminated sediments. Cloudy swelling of tubule cells has nevertheless been found as one of the most recurrent alterations in the kidneys of fish exposed to complex mixtures of contaminants (Triebskorn et al., 2004; Camargo and Martinez, 2007; Giari et al., 2007). According to our findings, it is possible to suggest a progressive series of tubule lesions according to the time of exposure: cloudy swelling of tubule epithelial cells; tubule cell necrosis; tubule disorganization leading to tubule disappearance. This pattern is consistent with the proliferative kidney disease (PKD) described in feral or laboratory-tested fish exposed to indiscriminated contaminant mixtures (e.g. Triebskorn et al., 2002). The relation between the observed renal lesions and the sediment contaminants may indicate that the damage should be linked to uptaken sediment organic contaminants (especially PAHs and PCBs) rather than being a response to overall contamination. The severe lesions observed in the tubules of body kidney (the secretory portion of fish kidneys) might be linked to the excretion of the highly reactive and toxic organic contaminant metabolites. Some of these metabolites (such as PAH o-quinones and diol epoxides) are formed by cytochrome monooxygenase systems to transform the hydrophobic compounds into more water soluble, thus more excretable, forms (Flowers-Geary et al., 1996; Burchiel et al., 2007). Flatfish have already been found, for instance, to rapidly catabolize PAHs (Varanasi and Gmur, 1981), which may contribute to a fast detoxification but also diffuse the highly toxic metabolites through the organism via the blood stream, eventually reaching the kidneys. Although the liver is considered to be the most important organ involved in contaminant metabolism, accumulation and excretion of xenobiotics and their metabolites are important renal functions (see Pritchard and Miller, 1997 for a review), a premise that is supported by the current findings. The severe PKD sustained by the body kidney of fish exposed to sediment C might contribute to explain the high mortality that occurred during this test. Nevertheless, much information is still lacking regarding the specific effects of xenobiotics, especially of organic compounds, in fish kidney.

In spite of its lower contaminant levels than sediment B, exposure to sediment C caused more severe damage to renal tubules than B. Still, both tests were responsible for more severe lesions in the chloride cells and body kidney that would be expected from the comparison between the measured sediment contaminants and the sediment quality guidelines. The current findings confirm that these guidelines are in essence a primary screening tool and should not make toxicity bioassays expendable, although they have been shown capable of predicting sediment toxicity in laboratorial studies (Long et al., 1998). It’s noteworthy, however, that the concentrations of some metallic contaminants in sediment B reach up to 3- and 4-fold the levels in sediment C, such as copper and zinc (both above the *PEL* thresholds). The results thus suggest that organic contaminants appear to have higher toxic effects

on osmotic balance mechanisms than metals. The toxicity effects observed, and more specifically the differences between tests B and C renal tubule lesions, might also be explained by the prevalence of some organic compounds in sediment C, such as total PCBs (reaching a 3-fold level in comparison to sediment B) and some PAHs, especially the higher molecular weight (more toxic) 4- and 5-ringed compounds. On the other hand, sediment handling prior to bioassay preparation and sediment resuspension caused by the animals' scavenging activities, may have promoted bioavailability of contaminants. In fact, bioturbation has been found responsible for increased availability of contaminants in the water column (Atkinson et al., 2007). Also, high levels of sediment fine particle fraction and organic matter favour contaminant retention in sediments but, combined with anoxia, enhance the release of contaminants into the water column during sediment disturbance events (Eggleton and Thomas, 2004), which is likely to have occurred in all tests, especially during exposures to sediments B and C. This toxicity magnification effect should not, however, explain on its own the differences between the kidney lesions observed in fish exposed to these sediments.

Exposure to moderate sediment-based contamination may cause severe lesions in organs involved in the maintenance of internal osmotic balance of juvenile *S. senegalensis*. Even though laboratorial bioassays may promote the release of xenobiotics adsorpted to sediment particles and organic fraction, it can be concluded that low to moderate sediment contamination, at least when associated to sediment disturbance events, may cause severe lesions in osmotic regulation structures in juvenile *S. senegalensis*. On the other hand, organic contaminants such as PAHs and PCBs appeared to have a more pronounced toxic effect on the mechanisms of osmotic balance than metallic elements and DDTs on tested fish. The results also confirm that, although a useful tool as a primary indicator of toxicity to biota, sediment quality guidelines should be complemented by the actual analysis of effects of contaminants to organisms.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by an FCT PhD grant (SFRH/BD/28465/2006). The authors would also like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and RNES (Reserva Natural do Estuário do Sado) for the logistic support and Pedro Pousão (Estação Piloto de Piscicultura, INRB/IPIMAR-Cripsul) for supplying the fish used in the present work.

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# Chapter 4. Simultaneous laboratory and *in situ* assays

## 4.1. Assessment of the genotoxic potential of contaminated estuarine sediments in fish peripheral blood: laboratory versus *in situ* studies[†]


### Abstract

Juvenile Senegalese soles (*Solea senegalensis*) were exposed to estuarine sediments through 28-day laboratory and *in situ* (field) bioassays. The sediments, collected from three distinct sites (a reference plus two contaminated) of the Sado Estuary (W Portugal) were characterized for total organic matter, redox potential, fine fraction and for the levels of metals, polycyclic aromatic hydrocarbons (PAHs) and organochlorines, namely polychlorinated biphenyls (PCBs) and dichloro diphenyl tricholoethane plus its main metabolites (DDTs). Genotoxicity was determined in whole peripheral blood by the single-cell gel electrophoresis (SCGE or "comet") assay and by scoring erythrocytic nuclear abnormalities (ENA). Analysis was complemented with the determination of lipid peroxidation in blood plasma by the thiobarbituric acid reactive substances (TBARS) protocol and cell type sorting. The results showed that exposure to contaminated sediments induced DNA fragmentation and clastogenesis. Still, exposure to the most contaminated sediment revealed a possible antagonistic effect between metallic and organic contaminants that was not observed for *in situ* exposures. The laboratory assay caused a more pronounced increase in ENA whereas a very significant increase in DNA fragmentation was observed in field-tested fish exposed to the reference sediment, which is possibly linked to increased lipid peroxidation that occurred, likely due to starvation. Influence of natural pathogens was ruled out by unaltered leukocyte counts. The statistical integration of data correlated lipid peroxidation with biological variables such as fish length and mass, whereas the genotoxicity biomarkers were more correlated to sediment contamination. It was demonstrated that laboratory and field bioassays for the risk assessment of sediment contamination may yield different genotoxicity profiles. Laboratory assays appear to enhance the potential of contamination by greatly increasing bioavailability. While field assays may provide more ecologically relevant data, the multiple environmental variables may produce sufficient background noise to mask the true effects of contamination.



### Keywords

DNA damage; Lipid peroxidation; Blood cell types; Peripheral blood; Sediment contamination; *Solea senegalensis*


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[†] Costa et al. (2011). *Environ. Res.* (doi:10.1016/j.envres.2010.09.011).

## 1. Introduction

Assessment of the genotoxic (mutagenic) potential of environmental contamination has been gaining growing attention within ecological monitoring, human occupational health and fundamental toxicology. One of the factors that account for the importance of determining the genotoxicity of xenobiotics is the relationship between mutagenesis and carcinogenesis. A number of techniques has been employed to study genotoxicity in a broad range of organisms, both *in vivo* and *in vitro*, including the micronucleus test and its variations (such as the scoring of total nuclear abnormalities), the sister chromatid assay, DNA-xenobiotic adduct formation and the SCGE, or "comet" assay as it is more commonly known, just to quote a few (refer to van der Oost et al., 2003, for a review on genotoxicity and other biomarkers for risk assessment of aquatic environments). Although the usefulness and adequacy of these techniques are seldom under debate, some authors have questioned their application in the monitoring of complex contaminant matrices as natural waters and sediments (e.g. Bombail et al., 2001; Wirzinger et al., 2007). The genotoxic potential of aquatic sediments is recognized as a primary area of concern for environmental toxicologists and, in spite of its inherent difficulties, a great number of tests have been developed to address this issue, using bacteria, invertebrates and fish (Chen and White, 2004). Little research, however, reports the potential differences between laboratory and field (*in situ*) assays. Different responses and sensitivity to environmental toxicity between the two types of assays with fish and aquatic invertebrates have already been reported and some authors argued that both are important for biomonitoring purposes (Hatch and Burton, 1999; Smolders et al., 2004), including those using flatfish as test organisms, a group of benthic vertebrates recognized as very sensitive to sediment-bound contamination (Johnson et al., 1998).

The two biomarkers, DNA strand breakage (given by the SCGE) and chromosome clastogenesis (assessed by the analysis of ENA); reflect different types of DNA damage. The SCGE assay quantitates DNA fragmentation, single- or double-strand, through the neutral or alkaline versions, respectively, a type of damage that may result from direct DNA chain oxidation, formation of xenobiotic-DNA adducts and alkali-labile sites. This sort of mutagenesis depends on the action of the cellular DNA-repairing machinery (since single-strand damage may be reversible); hence the genotoxic potential of xenobiotics may be masked by factors affecting cell physiology (see Sarasin, 2003, for a review). Conversely, clastogenesis involves errors during mitosis, causing deleterious mutations, aneuploidies, and other chromosome-level mutations that may be reflected in alterations of nuclear shape and induction of micronuclei. This type of damage is not repairable and is likely to more severely compromise cell and tissue viability. The SCGE assay has been regarded as a more sensitive, expedite and objective tool to assess mutagenesis than more traditional approaches such as sorting ENA. Still, the influence of many confounding factors such as DNA repair capability, subject health and others has been recognized as an important issue when considering this protocol for standard biomonitoring purposes (Møller et al., 2000). Due to the differences between the two biomarkers, the

employment of both has become a common procedure for genotoxicity assessment (e.g. Siu et al., 2004; Costa et al., 2008a; Neuparth et al., 2009).

Different contaminants give rise to distinct pathways of mutagenesis although it is believed that reactive oxygen species (ROS) are a common denominator in xenobiotic-induced DNA damage. Organic contaminants such as PAHs (among which is included the well-known carcinogen and teratogen benzo[a]pyrene) are known to induce genotoxicity through the activated PAH forms (e.g. epoxides and quinones) and ROS formed during PAH catabolism by the monooxygenase enzymes of the mitochondrial cytochrome machinery (refer to Miller and Ramos, 2001, for a review). Metals and metalloids, however, have a different, yet poorly understood, mutagenic action. Metalloid mutagenesis and carcinogenesis (such as for arsenic), for instance, have been linked to the formation of ROS (e.g. Liu et al., 2001), although the exact mechanism by which ROS are induced is unclear. It is also suspected that cadmium induces apoptosis and therefore DNA cleavage and clastogenesis, a process also depending on the formation of ROS (Risso-de Faverney et al., 2001). It is believed that metals and metalloids indirectly induce mutagenesis by impairing oxidative metabolism, e.g. by competing with ROS-scavenging thiols (reviewed by López-Barea and Gómez-Ariza, 2006). Other authors have found genotoxicity induced by UV light to be enhanced by mercury (Bradfield et al., 2006). The link between genotoxicity and ROS led to many toxicological studies involving genotoxicity and oxidative stress-related biomarkers, such as lipid peroxidation (e.g. Gagné et al., 2008; Moore et al., 2009; Grinevicius et al., 2009). Furthermore, blood cells and plasma are recognized as a convenient vector for analysis since peripheral blood reflects the global health status of the individual. Regarding this issue, fish blood has long gained special attention since fish red blood cells are nucleated and therefore suitable for ENA analysis and obtaining good nucleoids for the SCGE.

Estuaries are major areas of concern regarding environmental contamination. These are, however, complex and labile ecosystems typically subjected to many anthropogenic sources of contamination. The Sado Estuary (SW Europe) is no exception to this premise. It consists of a large estuarine basin (second largest in Portugal and one of the largest in Europe) that comprises the urban area of Setúbal, one of the densest heavy-industry conglomerates in the country, mariculture facilities and it also supports important fisheries for the local economy, tourism and part is classified as a natural reserve. The estuary is also subjected to river flows carrying pesticides and fertilizers from the extensive agriculture grounds upstream. For such reasons, recent projects have employed an ecotoxicological approach to assess sediment contamination risk in the area (Caeiro et al., 2005, Neuparth et al., 2005; Costa et al., 2008b), some of which already comprised laboratory bioassays with the benthic fish *Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae) (Costa et al., 2008a, 2009a, 2009b), a common fish in the estuary and of importance for local fisheries.

The surveyed sites from the Sado Estuary (Fig. 4.1.1) were selected according to previous research on the Sado Estuary that analyzed sediment physical characteristics and contaminant concentrations (see Caeiro et al., 2005; Costa et al., 2008a). Site R (taken as the reference site) is the farthest from direct pollution sources, especially effluent discharges, whereas sites $C_1$ and $C_2$ are

located next to the city of Setúbal t and at a newly-built harbour, respectively. These sites are also located just off the city's heavy-industry belt which includes shipyards, a thermoelectrical plant, a paper mill and chemical plants (including fertilizer industries), mineral ore deployment facilities and others, besides being subjected to urban effluents and runoffs from agriculture ground located upstream. Sediments from sites $C_1$ and $C_2$ have the highest organic and fine fraction loads and are also the most anoxic.

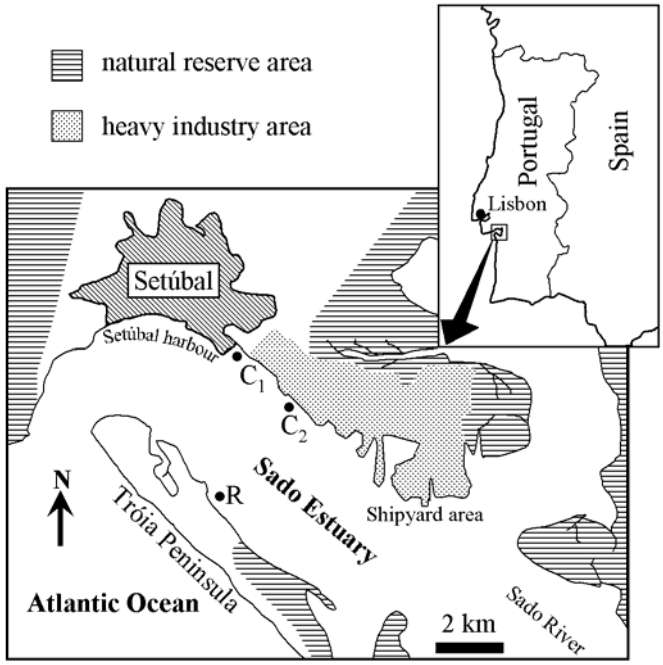


**Fig. 4.1.1.** Map of the study area showing the surveyed sites of the Sado Estuary: reference (R) and contaminated ($C_1$ and $C_2$).

The results from our previous assays performed with *S. senegalensis* showed that even moderate contamination levels have the potential of causing severe chronic lesions and alterations to fish. Also, the interactions organic and metallic contaminant interactions were likely responsible for antagonist and synergistic effects and responses of organisms to contamination. These results have shown that assessing sediment contamination has many constraints and, like noted by other authors, demand caution and a careful selection of bioassay procedures, biomarkers and test organisms (see Chapman et al., 2002, for an overview of the issues concerning the determination of sediment toxicity).

The present works aims at studying the differences and difficulties of determining the genotoxic potential of sediment-bound contamination between laboratory and field (*in situ*) bioassays. A contribution to the understanding of the interaction effects of distinct classes of metallic and organic contaminants on genotoxicity is also intended, integrating two genotoxicity biomarkers (determination of total DNA strand breakage and chromosomal clastogenesis), blood plasma lipid peroxides and peripheral blood cell counts.

## 2. Methods and materials

### *2.1. Experimental procedure*

The sediments (for assays and chemical analysis) were collected from the three sites (R, $C_1$ and $C_2$ - see Fig. 4.1.1) on May 2007 with a Petite Ponar grab. Laboratory and field assays were performed simultaneously during the same period. Juvenile laboratory hatched and reared Senegalese soles (standard length = 61.0 ± 8.4 mm; total wet weight = 3.1 ± 1.6 g), all from the same cohort, were used as test subjects. For simplification purposes, exposures to sediments collected from sites R, $C_1$ and $C_2$ will be throughout referred to as tests R, $C_1$ and $C_2$.

The laboratory assay was prepared by placing 2 L of fresh sediments in 15 L-capacity white polyvinyl tanks (with blunt edges) to which were added 10 L of clean, 0.45 μm-filtered, seawater. Sediments (providing a surface of ≈ 525 $cm^2$) were allowed to settle for 48 h before the beginning of the assay). A recirculation system was coupled to each tank, water flow being set to prevent sediment resuspension. Aeration was constant. The assays were performed in duplicate, with twenty animals being randomly distributed per tank. The assays were performed under the same conditions of the rearing systems: temperature = 18 ± 1 ºC, salinity = 32.1 ± 0.3, pH 8.0 ± 0.1, dissolved oxygen = 56.5 ± 0.2% and total ammonia was restrained within 1.6 ± 0.6 $mg.L^{-1}$. Water parameters were monitored weekly and a 25% water change was done at the same interval to ensure their constancy with minimal removal of contaminants and suspended matter. Fish were fed once a day with commercial M2 grade fish pellets (from Sorgal, Ovar, Portugal).

The field assay was performed with submerged cages (at a depth between 7 and 10 m), placed over the bottom using scuba equipment, ensuring that fish were in direct contact with the sediments. The cages (90 × 90 × 30 cm), lined with a 5 mm plastic mesh, were divided in two equal-sized compartments (replicates), each allocating twenty randomly-selected animals.

Both types of assays had the duration of 28 days, with sampling times scheduled at days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$). Five animals per replicate (ten at the total) were retrieved from the cages (and transported alive to the laboratory in separate 20 L-vessels per replicate) or test tanks at $T_{14}$ and $T_{28}$, measured for total wet weight ($ww_t$) and standard length ($L_s$). Blood was collected just above the lateral line, from each individual, with a hypodermic syringe previously washed with an EDTA solution to prevent clotting. Animals sampled at $T_0$ consisted of twelve fish collected directly from the rearing tanks and where handled as aforementioned.

### *2.2. Sediment analyses*

Sediment metallic elements, the non-metal selenium (Se), the metalloid arsenic (As) and the metals cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) were determined in dry sediment samples mineralized with a mixture of acids (6

mL HF 40% v/v to which was added 1 mL of the mixture 36% HCl plus 60% $HNO_3$ 3:1 v/v) in closed Teflon vials, according to Caetano et al. (2007). Element quantification was achieved by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series equipment. Total mercury (Hg) was determined in dried sediment samples by atomic absorption spectrometry (AAS) after pyrolysis of the samples at 750 ºC in an oxygen atmosphere in a combustion tube attached to an AMA-254 mercury analyzer (Leco), according to Costley et al. (2000). The reference sediments MESS-2and PACS-2 (National Research Council, Canada) and MAG-1 (US Geological Survey, USA) were analyzed by these procedures to validate the methods and the values were found within the certified range.

Sediment PAH concentrations were determined from dry samples spiked with surrogate standards (from Supelco) and Soxhlet-extracted with an acetone+hexane (1:1 v/v) mixture. Compounds were quantified by gas chromatography-mass spectrometry (GC-MS) as described by Martins et al. (2008), using a Finnigan GCQ system. A total of seventeen 3- to 6-ring PAHs were quantified. Organochlorines (18 PCB congeners and DDTs, namely *pp*'DDT plus the *pp*'DDD and *pp*'DDE metabolites) were quantified from dried sediment samples Soxhlet-extracted with *n*-hexane. Extracts were fractioned in a chromatographic column and compounds quantified by GC with an electron capture detector (Ferreira et al. 2003) using a Hewlett-Packard 6890 apparatus. Validation of the organic contaminant determination procedure was obtained by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the obtained values were observed to be within the certified range.

Sediment total organic matter (TOM) and was determined by organic carbon loss-on-ignition (LOI) at 500 ºC. Fine fraction (FF), particle size < 63 μm, was determined by hydraulic sieving after disaggregation with pyrophosphate. Both results are expressed as percentage of total sediment dry weight (dw). Sediment redox potential (Eh) was measured immediately after collection with an Orion 20A apparatus equipped with a H3131 platinum electrode with an Ag/AgCl reference electrode.

### *2.3. Analysis of the potential sediment contamination impact*

The sediments' potential for causing adverse biological effects of the tested sediments was evaluated by calculating the *PEL* quotient (*PEL-Q*) based on the published guideline values for coastal waters, namely the Threshold Effects Level (*TEL*) and the Probable Effects Level (*PEL*), according to MacDonald et al. (1996). The *PEL-Q* indice was calculated for each contaminant, according to the formula described by Long and MacDonald (1998):

$$PEL - Q_i = \frac{C_i}{PEL} \qquad [1]$$

Being *PEL* the guideline value for the contaminant *i* and $C_i$ the measured concentration of the

same contaminant. The Sediment Quality Guideline Quotient indice (*SQG-Q*), developed to compare sites affected by contaminant mixtures, was calculated for each sediment according to Long and MacDonald (1998) as:

$$SQG-Q = \frac{\sum_{i=1}^{n} PEL - Q_i}{n} \quad [2]$$

Where *n* is the total number of contaminants under analysis for which sediment quality guidelines are available. The three stations were sorted according to their sediment contamination potential for causing adverse biological effects: *SQG-Q* < 0.1 - unimpacted; 0.1 ≤ *SQG-Q* < 1 - moderately impacted; *SQG-Q* ≥ 1 - highly impacted (MacDonald et al., 2004).

*2.4. Determination of genotoxicity*

The alkaline single-cell gel electrophoresis assay was employed in whole-blood according to Singh et al. (1988) and as previously tested and described for this species by Costa et al. (2008a). Analysis was run immediately after blood collection to ensure maximum cell viability. All steps onward were performed under dim light to avoid accessory DNA strand breakage. The room temperature was held constant at ≈ 20 ºC and all solutions were used cold (≈ 4 ºC) to avoid DNA damage and agarose lifting from the slides. Blood cell suspensions were obtained by diluting (1:100) blood aliquots in cold PBS (phosphate-buffered saline), pH 7.4 with 0.7% NaCl. Aliquots of cell suspensions were then diluted in liquid (≈ 37 ºC) 1% w/v low-melting point agarose (Sigma) and placed (2 × 75 μL) on microscopy slides previously coated with dry high melting point agarose. After cell lysis for 1 h (in the dark, at 4 ºC) in a solution containing 2.64 % w/v NaCl, 3.72% w/v EDTA (w/v) and 5mM TRIS to which was added 10% v/v DMSO and 1% v/v Triton-X 100 just before use, slides were placed in cold alkaline electrophoresis solution (0.1 μM EDTA, 0.3 M NaOH) to promote DNA unwinding and high expression of alkali-labile sites. Electrophoresis was run in the dark and in cold for 30 min at 25 V using a Bio-Rad Sub-Cell 96 apparatus. Slides were afterwards neutralized in Tris-HCl buffer (pH 7.5). Slides were stained with ethidium bromide for analysis. Approximately 100 nucleoids were scored per slide, the % of DNA in the comets' tail being used as a direct measure of total (single and double strand) DNA strand-breakage (Lee and Steinert, 2003). Image analysis was performed with the software CometScore 1.5 (TriTek Corp.).

Erythrocyte nuclear clastogenesis was determined by scoring the percentage of mature red blood cells (RBCs) exhibiting nuclear abnormalities (ENA). The analysis was performed on methanol--fixed blood smears (15 min), stained with 0.1 $g.L^{-1}$ acridine orange fluorochrome, as described by Costa and Costa (2007). Approximately 1,000 mature erythrocytes were scored per slide.

For both analyses, a Leica DMLB model microscope adapted for epifluorescence and

equipped with an EL6000 light source for mercury short-arc reflector lamps was used. I3 and N2.1 filters were employed for acridine orange- and ethidium bromide-stained slides, respectively. All equipment was obtained from Leica Microsystems. Total DNA strand breakage and ENA data are expressed as variation coefficients ($VC_{TSB}$ and $VC_{ENA}$, respectively) relatively to $T_0$ animals.

*2.5. Discrimination of blood cell types*

The relative proportions of leukocytes, thrombocytes and immature erythrocytes were determined in acridine orange-stained blood smears, prepared as described in the previous section. At least 1,000 intact cells were scored per slide. Results are given as percentage of each cell class relatively to the total number of cells counted.

*2.6. Plasma lipid peroxides assay*

The determination of blood plasma lipid peroxides was adapted from the thiobarbituric acid reactive substances (TBARS) protocol developed by Uchiyama and Mihara (1978). In brief: blood samples were centrifuged (10 min, 10,000 × *g*) to separate the plasma from the blood cells and five microlitres of plasma were diluted in 45 μL PBS (pH 7.4, with 0.7 % NaCl) to which was added 100 μL ice-cold trichloroacetic acid (10% m/v). Samples were afterwards incubated at ≈ 4 ºC for 15 min to allow protein to precipitate. Following a 15 min centrifugation (2,200 × *g*), 100 μL of the clear supernatant was taken and added 100 μL 0.1 % m/v thiobarbituric acid (diluted in MQ-grade ultrapure water by adding 10M NaOH). The samples were then incubated for 15 min on boiling water. The absorbance of the reddish pigment (530 nm) was measured using a Benchmark Microplate Reader (Bio-Rad). Ninety six-well plates were used, each well containing 150 μL of the reaction, blanks or standards. Quantification was performed through an eight-point calibration curve (0-300 nM TBARS) using malondialdehyde bis(dimethylacetal) from Merck as standard. Preliminary set-up TBARS assays confirmed that residual haemoglobin produces false MDA-equivalent positives, which was observed to be highly relevant when separating plasma from the small blood samples obtained from the fish (e.g. see Gilbert et al., 1984 concerning the interference of haemolysates on TBARS test). For such reason, a correction factor was introduced according to the relative proportion of residual haemoglobin present in plasma samples, determined by the cyanmethemoglobin method (van Kampen and Zijlstra, 1961). Final results are expressed as plasma lipid peroxide variation coefficients relatively to $T_0$-sampled fish ($VC_{TBARS}$).

*2.7. Statistical analyses*

Following the invalidation of the homogeneity of variances assumption (through the Levene's test), non-parametric analyses were done using the Kruskall-Wallis ANOVA by ranks *H* statistic for

overall differences, the Mann-Whitney *U* test for pairwise comparisons and the Spearman's rank-order correlation *R*. Correlation-based principal component analysis (PCA) was employed to rank the variables that contribute the most to explain the variation within laboratory and field assays. Discriminant analysis was used to assess the divergence between both types of assays. All statistics were computed using Statistica (Statsoft Inc.).

## 3. Results

### *3.1. Mortality*

A distinct pattern of mortality was observed between the two types of assays. In the laboratory assays, exposure to sediment $C_2$ yielded the highest level of mortality after the 28 days of the assay (14 out of 40 individuals), followed by exposed to sediment R and $C_1$ (7/40 and 5/40, respectively). With respect to the field assay, exposure to sediment R (the reference sediment) caused the highest mortality at the end of the experiment (13/40) while only 5/40 fish were lost for both tests $C_1$ and $C_2$.

### *3.2. Sediment characterization*

The three sediments revealed distinct physical characteristics and different levels of contamination (Table 4.1.1). The sediment from site $C_1$ was found to be the most contaminated by metals, although presenting PAH levels close to those observed for sediment $C_2$, the most contaminated by 4- to 6-ring compounds, except Benzo[e]pyrene. The reference sediment (from site R) was found to be unimpacted, presenting the lowest global sediment quality guideline quotient (*SQG-*$Q_t$). Sediments $C_1$ and $C_2$ were, however, found to be globally moderately impacted by contaminants (*SQG-*$Q_t$ = 0.33 and *SQG-*$Q_t$ = 0.28, respectively). Copper is the only contaminant reaching the *TEL* guideline value in sediment from site R, confirming its overall low contamination. On the other hand, *PEL* levels are reached for Cu, Zn and fluoranthene in sediments from site $C_1$ and total Hg (organic plus inorganic species) and fluoranthene in sediments from site $C_2$, hence the major contributors to the *SQG-Q* levels observed. The sediment from site $C_1$ had the highest content in TOM and FF, followed by $C_2$. As expected, a linear relationship was observed between FF and TOM ($r^2$ = 0.98). The most anoxic sediment was found to be from site $C_2$ (with the lowest Eh), followed by $C_1$. The phenanthrene/anthracene and fluoranthene/pyrene ratios were found to be > 1 and < 10, respectively, for all sediments, indicating the PAHs are essentially of pyrolytic nature (combustion--derived) and not petrogenic (Budzinski et al., 1997).

*3.3. Genotoxicity*

Total DNA fragmentation analyzed by the SCGE assay yielded distinct TSB levels between laboratory- and field-exposed fish. Only in laboratory-tested fish collected at $T_{14}$ and exposed to the reference sediment (R) no significant induction of total DNA strand breakage was observed relatively to $T_0$ fish (Mann-Whitney *U*, $p > 0.05$). All other tests caused an increase in TSB, ranging between 1.5-fold (in laboratory-exposure to the reference sediment at $T_{28}$) and 5.8-fold (in fish exposed to the $C_2$ sediment, in laboratory, collected at $T_{28}$), relatively to $T_0$ animals (Fig. 4.1.2). Laboratory exposure to sediment $C_2$ was the responsible for the highest induction of TSB, with significant differences being found from all other tests, at $T_{14}$ and $T_{28}$ (Mann-Whitney *U*, $p < 0.01$). Laboratory exposure to sediment $C_1$ derived a significant increase in $VC_{TSB}$ from $T_{14}$ to $T_{28}$, which was not observed for the other tests. Exposure to sediment $C_1$ caused the highest induction of TSB in the field assay, statistically different from exposures to sediments R and $C_2$ at $T_{14}$ and only from exposures to sediment $C_2$ at $T_{28}$ (Mann-Whitney *U*, $p < 0.01$), caused by a significant increase in TSB in R-tested fish from $T_{14}$ to $T_{28}$ (Mann-Whitney *U*, $p < 0.01$). No differences were found between replicates (Kruskall-Wallis *H*, $p > 0.05$).

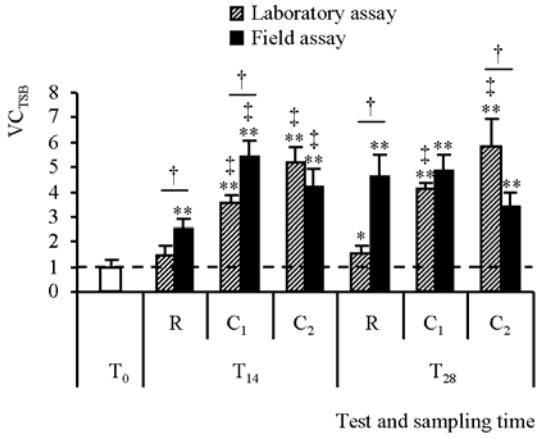


**Fig. 4.1.2.** Variation coefficients of total DNA strand breakage ($VC_{TSB}$) in whole-blood of tested individuals in relation to $T_0$ animals (dashed line), as determined by SCGE. * and ** mean significant differences from $T_0$ fish (Mann-Whitney *U*), $p < 0.05$ and $p < 0.01$, respectively. ‡ means significant differences to reference test (exposure to sediment R) at respective sampling time and assay type (Mann-Whitney *U*, $p < 0.01$). † means significant differences between laboratory- and field-exposed fish (Mann-Whitney *U*, $p < 0.01$). Error bars represent 95% confidence intervals.

In accordance to what has been previously described for the species (Costa et al., 2008a), nuclear budding and fragmentation were the most recurrent ENA observed (Fig. 4.1.3b-e). Analysis of ENA depicted significant differences between laboratory- and field-exposed fish (Kruskall-Wallis *H*, $p < 0.01$) since, even though a similar pattern was observed regarding the induction of ENA relatively

to $T_0$, the field-exposed fish exhibited lower frequencies of mature erythrocytes with evidence of clastogenesis (Fig. 4.1.4). Laboratory exposure to sediment $C_2$ induced more ENA than the other tests (Mann-Whitney *U*, $p < 0.01$). Regarding the field assay, tests $C_1$ and $C_2$ did not exhibit significant differences between each other at either $T_{14}$ or $T_{28}$ (Mann-Whitney *U*, $p > 0.14$ and $p = 0.43$, respectively), although both significantly induced clastogenesis comparatively to exposure to the reference sediment at both $T_{14}$ and $T_{28}$ (Mann-Whitney *U*, $p < 0.05$). Still, no increase was observed from $T_{14}$ to $T_{28}$ for any field test (Mann-Whitney *U*, $p > 0.05$), whereas in the laboratory assay the opposite was observed, including in test R. Unlike the laboratory assay, field exposure to the reference sediment did not cause any significant increase in clastogenesis relatively to $T_0$ at $T_{14}$ or even at $T_{28}$ (Mann-Whitney *U*, $p > 0.05$). No differences were found between replicates (Kruskall-Wallis *H*, $p > 0.05$).

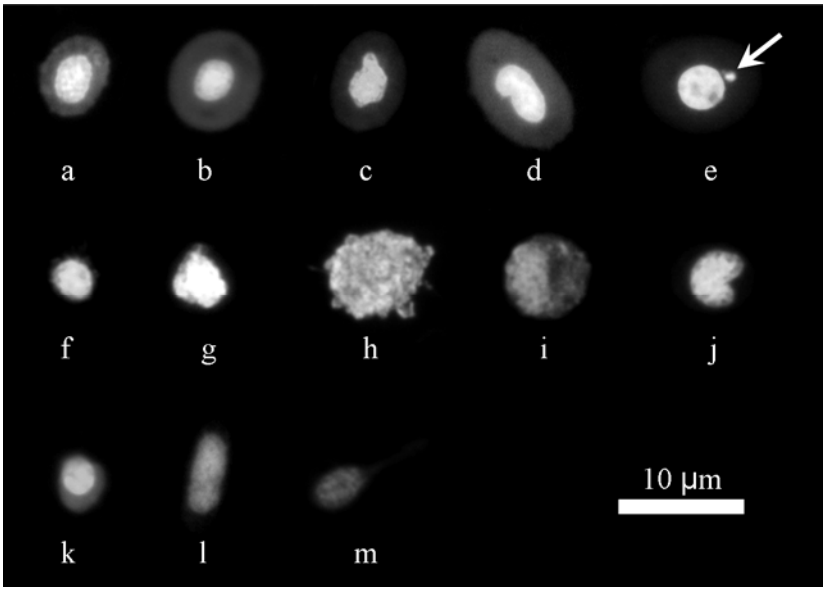


**Fig. 4.1.3.** Representative micrographs of observed cell types and alterations. a-e) erythrocytes: (a) normal immature erythrocyte; b) normal mature erythrocyte; c-e) mature erythrocytes exhibiting nuclear abnormalities such as (c) budding nucleus, (d) bilobed (fragmenting) nucleus and (e) micronucleus (arrow). f-j) leukocytes: f--h) different sized lymphocytes; i) neutrophil and j) monocyte. k-m) thrombocytes: (k) round; (l) rectangular (the most common sort) and (m) spindle-shaped.

**Table 4.1.1.** Physico-chemical characterization and contamination profiles of the sediments collected from the three study sites (R, $C_1$ and $C_2$). The *TEL* and *PEL* sediment quality guidelines were obtained from Macdonald et al. (1996).

| | | | | Site | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | **R** | **$C_1$** | **$C_2$** | | |
| | | | TOM (%) | 2 | 10 | 7 | | |
| | | | FF (%) | 23 | 96 | 76 | | |
| | | | Eh (mV) | -140 | -300 | -312 | *SQGs* | |
| Contaminant | | | | | | | *TEL* | *PEL* |
| | Element (µg.g$^{-1}$ sediment dw) | | As | 5.20 ± 0.10 | 23.98 ± 0.48* | 20.69 ± 0.41* | 7.24 | 41.6 |
| | | | Cd | 0.06 ± 0.00 | 0.26 ± 0.01 | 0.29 ± 0.01 | 0.68 | 4.21 |
| | | | Co | 3.37 ± 0.07 | 13.94 ± 0.28 | 9.43 ± 0.19 | *NG* | *NG* |
| | | | Cr | 18.14 ± 0.36 | 80.73 ± 1.61* | 51.70 ± 1.03 | 52.3 | 160 |
| | | | Cu | 28.20 ± 0.56* | 172.72 ± 3.45** | 95.31 ± 1.91* | 18.7 | 108 |
| | | | Hg | 0.11 ± 0.00 | 0.69 ± 0.01* | 0.71 ± 0.01** | 0.13 | 0.7 |
| | | | Mn | 100.75 ± 2.01 | 464.34 ± 9.29 | 362.47 ± 7.25 | *NG* | *NG* |
| | | | Ni | 7.31 ± 0.15 | 33.30 ± 0.67* | 20.49 ± 0.41* | 15.9 | 42.8 |
| | | | Pb | 18.57 ± 0.37 | 55.19 ± 1.10* | 43.76 ± 0.88* | 30.2 | 112 |
| | | | Se | 0.27 ± 0.01 | 1.21 ± 0.02 | 0.80 ± 0.02 | *NG* | *NG* |
| | | | Zn | 72.29 ± 1.45 | 364.83 ± 7.30** | 269.31 ± 5.39* | 124 | 271 |
| | | | *SQG-Q Metallic/Element* | **0.16** | **0.79** | **0.58** | | |
| Organic (ng.g$^{-1}$ sediment dw) | PAH | 3-ring | Acenaphthylene | 0.79 ± 0.02 | 2.38 ± 0.05 | 2.18 ± 0.04 | 5.87 | 128 |
| | | | Acenaphthene | 0.73 ± 0.01 | 12.25 ± 0.24* | 7.83 ± 0.16* | 6.71 | 88.9 |
| | | | Fluorene | 1.19 ± 0.02 | 15.33 ± 0.31 | 9.95 ± 0.20 | 21.2 | 144 |
| | | | Phenanthrene | 10.28 ± 0.21 | 63.87 ± 1.28 | 59.91 ± 1.20 | 86.7 | 544 |
| | | | Anthracene | 2.30 ± 0.05 | 21.00 ± 0.42 | 20.84 ± 0.42 | 46.9 | 245 |
| | | 4-ring | Fluoranthene | 23.34 ± 0.47 | 315.71 ± 6.31* | 345.24 ± 6.90* | 113 | 1,494 |
| | | | Pyrene | 21.51 ± 0.43 | 263.18 ± 5.26* | 286.33 ± 5.73* | 153 | 1,398 |
| | | | Benzo[a]anthracene | 3.70 ± 0.07 | 81.25 ± 1.62* | 93.99 ± 1.88* | 74.8 | 693 |
| | | | Chrysene | 2.35 ± 0.05 | 41.06 ± 0.82 | 46.68 ± 0.93 | 108 | 846 |
| | | 5-ring | Benzo[b]fluoranthene | 5.71 ± 0.11 | 98.00 ± 1.96 | 115.97 ± 2.32 | *NG* | *NG* |
| | | | Benzo[k]fluoranthene | 2.22 ± 0.04 | 30.76 ± 0.62 | 44.82 ± 0.90 | *NG* | *NG* |
| | | | Benzo[e]pyrene | 4.80 ± 0.10 | 74.95 ± 1.50 | 8.96 ± 0.18 | *NG* | *NG* |
| | | | Benzo[a]pyrene | 5.42 ± 0.11 | 101.86 ± 2.04* | 126.76 ± 2.54* | 88.8 | 763 |
| | | | Perylene | 7.83 ± 0.16 | 96.29 ± 1.93 | 113.47 ± 2.27 | *NG* | *NG* |
| | | | Dibenzo[a,h]anthracene | 0.66 ± 0.01 | 13.32 ± 0.27* | 13.93 ± 0.28* | 6.22 | 135 |
| | | 6-ring | Indeno[1,2,3-cd]pyrene | 5.06 ± 0.10 | 82.06 ± 1.64 | 95.00 ± 1.90 | *NG* | *NG* |
| | | | Benzo[g,h,i]perylene | 3.91 ± 0.08 | 51.93 ± 1.04 | 55.85 ± 1.12 | *NG* | *NG* |
| | | | **tPAH** | **101.8 ± 2.0** | **1,365.20 ± 27.3** | **1,447.7 ± 29.0** | **1,684** | **16,770** |
| | | | *SQG-Q PAHs* | **0.02** | **0.11** | **0.12** | | |
| | PCB | | 18 | < d.l. | 0.27 ± 0.01 | 0.42 ± 0.01 | *NG* | *NG* |
| | | Trichlorinated | 26 | < d.l. | 1.80 ± 0.04 | 1.99 ± 0.04 | *NG* | *NG* |
| | | | 31 | 0.05 ± 0.00 | 0.26 ± 0.01 | 0.34 ± 0.01 | *NG* | *NG* |
| | | | 44 | 0.03 ± 0.00 | 0.17 ± 0.00 | 0.40 ± 0.01 | *NG* | *NG* |
| | | Tetrachlorinated | 49 | < d.l. | 0.13 ± 0.00 | 0.24 ± 0.00 | *NG* | *NG* |
| | | | 52 | < d.l. | 0.10 ± 0.00 | 0.44 ± 0.01 | *NG* | *NG* |
| | | | 101 | 0.03 ± 0.00 | 0.25 ± 0.00 | 0.46 ± 0.01 | *NG* | *NG* |
| | | Pentachlorinated | 105 | < d.l. | 0.26 ± 0.01 | 0.39 ± 0.01 | *NG* | *NG* |
| | | | 118 | < d.l. | 0.55 ± 0.01 | 0.77 ± 0.02 | *NG* | *NG* |
| | | | 128 | < d.l. | 0.26 ± 0.01 | 0.48 ± 0.01 | *NG* | *NG* |
| | | | 138 | 0.14 ± 0.00 | 0.71 ± 0.01 | 1.08 ± 0.02 | *NG* | *NG* |
| | | | 149 | 0.08 ± 0.00 | 0.05 ± 0.00 | 0.30 ± 0.01 | *NG* | *NG* |
| | | Hexachlorinated | 151 | 0.06 ± 0.00 | 0.77 ± 0.02 | 1.14 ± 0.02 | *NG* | *NG* |
| | | | 153 | 0.13 ± 0.00 | 0.98 ± 0.02 | 1.28 ± 0.03 | *NG* | *NG* |
| | | | 170 | 0.07 ± 0.00 | 0.20 ± 0.00 | 0.25 ± 0.01 | *NG* | *NG* |
| | | | 180 | 0.05 ± 0.00 | 0.73 ± 0.01 | 1.22 ± 0.02 | *NG* | *NG* |
| | | Heptachlorinated | 187 | 0.14 ± 0.00 | 0.29 ± 0.01 | 0.47 ± 0.01 | *NG* | *NG* |
| | | | 194 | 0.01 ± 0.00 | 0.12 ± 0.00 | 0.29 ± 0.01 | *NG* | *NG* |
| | | | **tPCB** | **0.80 ± 0.02** | **7.91 ± 0.16** | **11.97 ± 0.24** | **21.6** | **189** |
| | | | *SQG-Q PCBs* | **0.00** | **0.04** | **0.06** | | |
| | DDT | | *pp*'DDD | < d.l. | 0.37 ± 0.01 | 0.71 ± 0.01 | 1.22 | 7.81 |
| | | | *pp*'DDE | < d.l. | < d.l | 0.59 ± 0.01 | 2.07 | 374 |
| | | | *pp*'DDT | < d.l. | < d.l | 1.22 ± 0.02* | 1.19 | 4.77 |
| | | | **tDDT** | **< d.l** | **0.37 ± 0.01** | **2.52 ± 0.05** | **3.89** | **51.7** |
| | | | *SQG-Q DDTs* | **0.00** | **0.02** | **0.12** | | |
| | | | *SQG-Q Organic* | **0.02** | **0.09** | **0.11** | | |
| | | | *SQG-Q Total* | **0.06** | **0.33** | **0.28** | | |
| | | | Impact rating | *Unimpacted* | ***Moderate*** | ***Moderate*** | | |

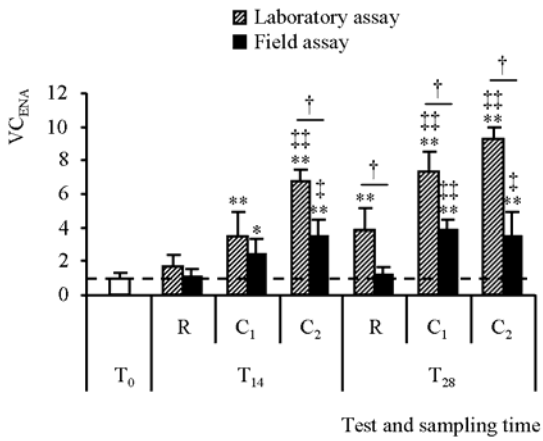


**Fig. 4.1.4.** Variation coefficients of the percentage of mature red blood cells exhibiting erythrocytic nuclear abnormalities ($VC_{ENA}$) comparatively to $T_0$ animals (dashed line). * and ** mean significant differences from $T_0$ fish (Mann-Whitney *U*), $p < 0.05$ and $p < 0.01$, respectively. ‡ and ‡‡ mean significant differences to reference test (exposure to sediment R) at respective sampling time and assay type (Mann-Whitney *U*, $p < 0.05$ and $p < 0.01$, respectively). † means significant differences between laboratory- and field-exposed fish (Mann--Whitney *U*, $p < 0.01$). Error bars represent 95% confidence intervals.

*3.4. Blood plasma lipid peroxidation*

Only field exposure to the tested sediments produced a significant increase in plasma lipid peroxides (inferred from TBARS variation) comparatively to $T_0$ fish and no significant differences were found between tests $C_1$ and $C_2$ to test R (reference) for either type of assay (Fig. 4.1.5). However, whereas field-exposures to sediments $C_1$ and $C_2$ caused a significant increase in $VC_{TBARS}$ from $T_0$ to $T_{14}$, exposure to the reference sediment depicted the most significant increase from $T_0$ fish but after 28 days of exposure (Mann-Whitney *U*, $p < 0.01$), although no differences were found for R-tested fish (in the field) between $T_{14}$ and $T_{28}$. No differences were found between replicates (Kruskall-Wallis *H*, $p > 0.05$).

*3.5. Quantification of blood cell types*

The percentages of immature erythrocytes (Fig. 4.1.3a), leukocytes (Fig. 4.1.3f-j) and thrombocytes (Fig. 4.1.3k-m) were found to be highly variable within all tested fish (Fig. 4.1.6). Exposure to the reference sediment caused a significant decrease in total leukocyte counts, at $T_{14}$ (field assay) and $T_{28}$ (laboratory assay). Non-lymphocyte leukocyte counts were found to be very low, regardless of test (≈ 0.1 - 0.5 %), hence alterations in leukocyte numbers were mostly contributed by lymphocytes. At $T_{28}$, in laboratory-tested fish, the leukocyte percentage was found to be significantly higher in fish exposed to sediment $C_2$ comparatively to R- and $C_1$-tested fish. (Mann-Whitney *U*, $p < 0.01$). In the field assays, again at $T_{28}$, both exposures to $C_1$ and $C_2$ produced significant higher leukocyte proportions comparatively to test R (Mann-Whitney *U*, $p < 0.05$ and $p < 0.01$, respectively)

but no significant differences were found between tests $C_1$ and $C_2$ and no differences were found between laboratory and field-tested fish regarding these two tests. No significant differences were found for other cell types. No differences were found between replicates (Kruskall-Wallis *H*, $p > 0.05$).

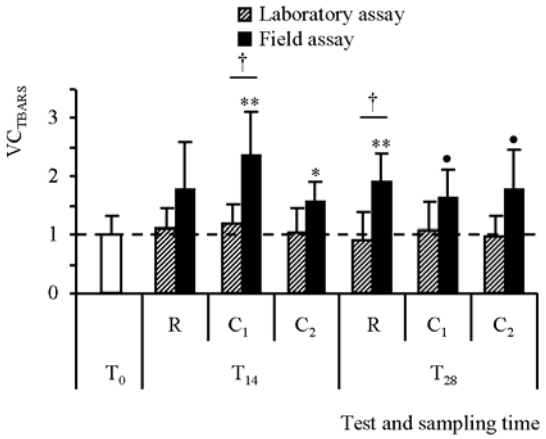


**Fig. 4.1.5.** Variation coefficients of lipid peroxide-like substances ($VC_{TBARS}$) in blood plasma relatively to $T_0$ animals (dashed line), as determined by the thiobarbituric acid-reactive substances test. •, * and ** mean significant differences from $T_0$ fish (Mann-Whitney *U*), $p < 0.10$, $p < 0.05$ and $p < 0.01$, respectively. † means significant differences between laboratory- and field-exposed fish (Mann-Whitney *U*, $p < 0.01$). Error bars represent 95% confidence intervals.

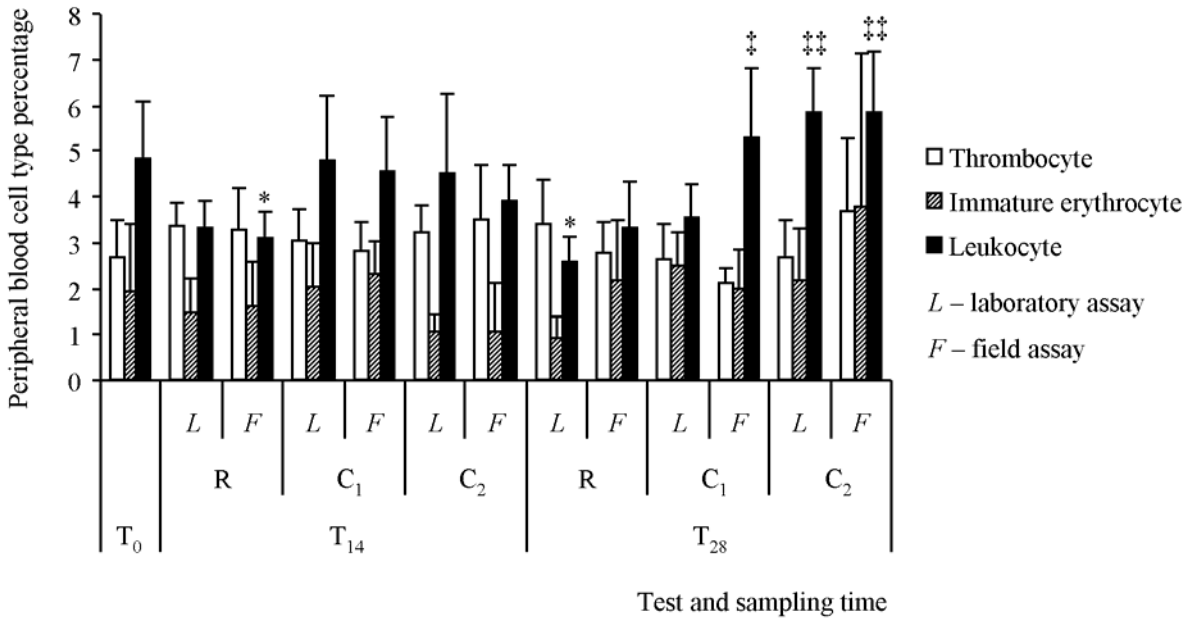


**Fig. 4.1.6.** Average thrombocyte, immature erythrocyte and leukocyte percentages in fish exposed to sediments from stations R, $C_1$ and $C_2$. * means differences from $T_0$-fish, $p < 0.05$ (Mann-Whitney *U*). ‡ and ‡‡ mean significant differences to reference test (exposure to sediment R) at respective sampling time and assay type (Mann-Whitney *U*, $p < 0.05$ and $p < 0.01$, respectively). Error bars represent 95% confidence intervals.

*3.6. Statistical integration of data*

Principal component analysis comprising all biological plus *SQG-Q*s (for total, metallic, organic, PAH, PCB and DDT contamination) retrieved the global model for laboratory-tested fish as the one explaining the highest percentage of total observed variance, followed by the laboratory+field- and field-tested fish models (cumulative explained variance on factors 1 and 2 was 63, 60 and 57%, respectively). For all models, the genotoxicity variation coefficients ($VC_{ENA}$ and $VC_{TSB}$), together with the percentage of leukocytes in blood, appeared more strongly correlated to the sediment quality guideline quotients (*SQG-Qs*), while a distinct group comprises the size-related variables ($L_s$ and $ww_t$), the variation coefficients of TBARS ($VC_{TBARS}$) and the percentage of thrombocytes (Fig. 4.1.7). However, $VC_{ENA}$ and $VC_{TSB}$ were found to provide a greater contribution to the laboratory assay's PCA than in the field assay's (0.088, 0.058 and 0.025 and 0.061 on factor 1, respectively), as well as they reverse their relative importance. On the other hand, the percentage of immature erythrocytes is better correlated with fish size in the field assay's PCA and with the sediment contamination indices in the laboratory assay's. The segregation of sampled animals (all biological variables) according to sampling time ($T_{14}$ versus $T_{28}$) by means of discriminant analysis reveals a better separation of laboratory-, field-tested and reared animals (fish collected at $T_0$) after 28 days of exposure, which is translated into the lowest Wilks' $\lambda$ and percentage of incorrect case classifications (Fig. 4.1.8).

**4. Discussion and conclusions**

Ecological risk assessment of contaminated sediments is a difficult task not only due to the likely occurrence of antagonist and synergistic effects drawn by mixtures of contaminants but also to the complex physico-chemical nature of the sediments themselves and its consequence on bioavailability. The integration of toxicity responses and effects in organisms with sediment quality guidelines and respective indices for contaminant mixtures (the *SQG-Q*s employed in the current work) should provide a more realistic approach to estimate the probability of occurring adverse affects to organisms than simple xenobiotic concentrations in the sediments since these indices are based on large datasets from multiple organisms, sediment types and contaminant classes (Long and MacDonald, 1998; Chapman et al., 2002). The present findings suggest that sediments $C_1$ and $C_2$, found to be overall moderately contaminated according to *SQG-Q*s, induced genotoxicity in both laboratory and field assays. However, the two types of bioassays yielded different patterns of genotoxicity, oxidative stress status and even mortality, which are likely caused by differences in the assays' conditions that affect bioavailability and overall fish stress and health status.

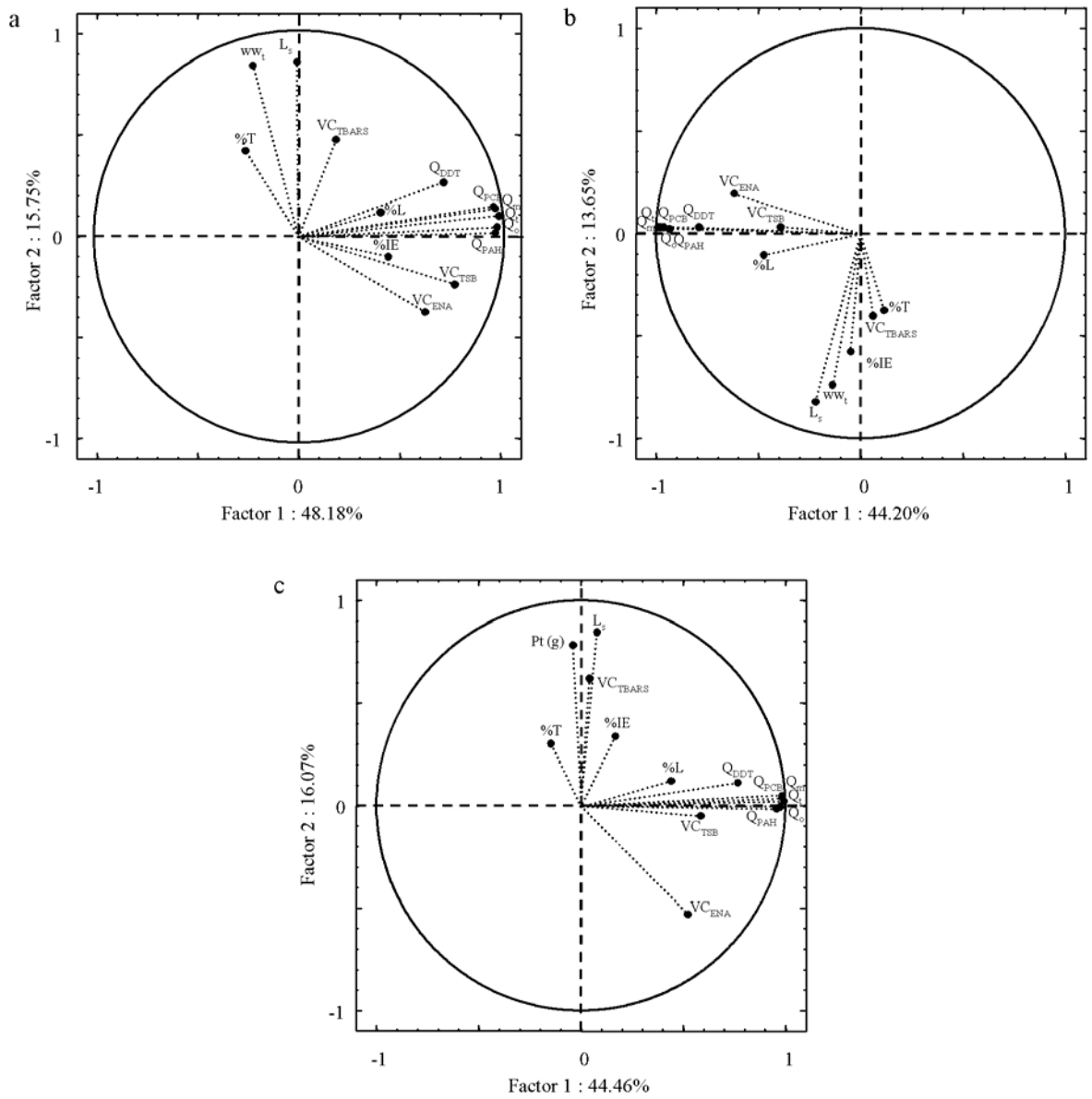


**Fig. 4.1.7.** Scatterplot of the principal component analysis (PCA) results (combination of $T_{14}$- and $T_{28}$- collected fish). a) Laboratory assay (factor 1 eigenvalue = 6.74; factor 2 eigenvalue = 2.20); b) field assay (factor 1 eigenvalue = 6.19; factor 2 eigenvalue = 1.91) and c) global PCA, combining laboratory- and field-tested fish (factor 1 eigenvalue = 6.22; factor 2 eigenvalue = 2.25). %IE, percentage of immature erythrocytes; %L, percentage of leukocytes; %T, percentage of thrombocytes; $Q_{DDT}$, *SQG-Q* for DDTs; $Q_m$, *SQG-Q* for inorganic (metal, metalloid and non-metal) contaminants; $Q_o$, *SQG-Q* for organic contaminants; $Q_{PAH}$, *SQG-Q* for PAHs; $Q_{PCB}$, *SQG-Q* for PCBs; $Q_t$, *SQG- -Q* for total contaminants; $VC_{ENA}$, $VC_{TBARS}$ and $VC_{TSB}$, variation coefficients for erythrocytic nuclear abnormalities, thiobarbituric acid reactive substances and total DNA strand breakage, respectively.

Comparing the levels of contaminants present in the tested sediments (collected in the spring of 2007) to the levels obtained in previous research at the same locations during the preceding autumn (Costa et al., 2008a, 2009a, 2009b) it may be inferred that the contaminant concentrations are within the expected order of magnitude. Still, a considerable rise in the levels of metals has occurred from autumn to spring regarding sediment $C_2$, especially regarding Cr and Zn, more than two- and three-

-fold, respectively. Conversely, PAH levels have moderately increased from the previous survey in sediments $C_1$ and $C_2$ (caused especially by an increase by almost the double of the concentrations of fluoranthene and pyrene), although $C_2$ remained the most contaminated by these substances and PCBs, within a similar scale of contamination. The reference sediment's levels of contamination decreased for all classes of xenobiotics, from autumn to spring. Nevertheless, sediment $C_1$ retains the status of the most contaminated and, according to the analysis of *SQG-Q*s, the probability of causing adverse effects may rank the tested sediments in the order: $R < C_2 < C_1$ for element contaminants and total contamination and $R < C_1 < C_2$ for organic xenobiotics. Still, PAHs are the major xenobiotic class of concern among organic contaminants since organochlorines are overall poorly represented. The observed alterations in the sediments' contaminant levels from autumn to spring sampling may be explained by variation in grain size, seasonality (influencing e.g. freshwater intake), natural depositing of contaminants that are continuously being released from pollution hotspots. Point local variations in sediment geochemistry may have a significant weight, such as the fact that the new site $C_2$ is located inside a recent harbour that has been subjected to dredging and settlement of a new sediment layer. In general, however, the results from sediment chemistry are in accordance with the ecological risk determined for the study area from past research (Caeiro et al., 2009).

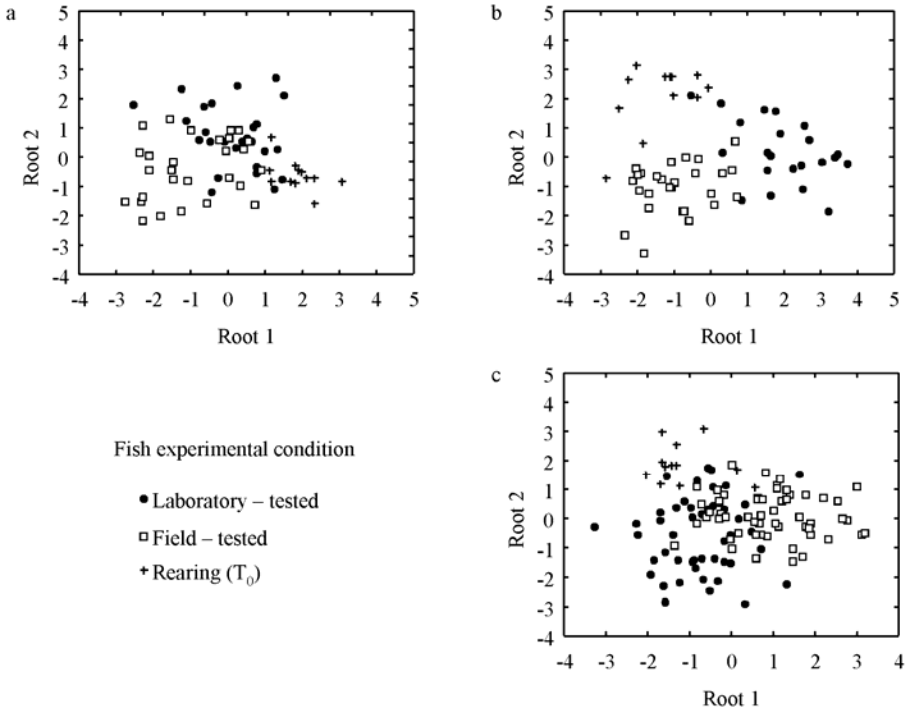


**Fig. 4.1.8.** Discriminant analysis scatterplots, all-biological variable models combining fish exposed to the three tested sediments. a) Fish collected at $T_{14}$ plus $T_0$, (Wilks' $\lambda$ = 0.35, 26% incorrect classifications); b) Fish collected at $T_{28}$ plus $T_0$ (Wilks' $\lambda$ = 0.13; 13% incorrect classifications) and c) global model comprising animals collected at all sampling times (Wilks' $\lambda$ = 0.36; 24% incorrect classifications).

In spite of the differences observed concerning the concentrations of some metals and organic compounds, DNA fragmentation and chromosomal clastogenesis depicted a similar pattern to our previous work with laboratory exposure to sediments from proximate locations (see Costa et al.,

2008a). Still, different patterns between field and laboratory assays were observed, especially regarding the genotoxicity biomarkers (ENA and TSB) and lipid peroxides in plasma. The differences may be explained by two sets of factors: i) factors that potentially enhanced bioavailability of contaminants, increasing the toxicity of sediment-bound xenobiotics in the laboratory and enhancing the effects of their interactions and ii) environmental factors that influenced genotoxicity and lipid peroxidation (mainly by predisposing oxidative stress) in field-tested fish.

Sediment organic matter and fine particle fraction operate as traps for contaminants. When subjected to disturbance, especially when combined with oxic-anoxic shifting, xenobiotics sorpted to organic matter- and fine fraction-rich sediments may be transferred to the water column, increasing their bioavailability, especially if a shift in the redox/pH status occurs, for instance, due to access of dissolved oxygen (see Eggleton and Thomas, 2004). It is thus likely that sediment collection and handling during the preparation of the laboratory assays favoured the release of xenobiotics from the sediment matrix to the water by a combination of disturbance upon collecting and handling, and also animal-driven resuspension, and the air flow that continuously oxygenated the water and upper layer of sediments, thus contributing to the change of its redox status. This may contribute to explain why ENA suffered a greater increase in laboratory-tested fish comparatively to $T_0$ animals (which reflect the standard rearing conditions). Still, genotoxicity resulting from laboratory exposures was higher regarding exposure to sediment $C_2$, the sediment most contaminated by PAHs but not the globally most impacted. However, exposure to sediment $C_1$ (the most impacted) caused the only significant increase in TSB from $T_{14}$ to $T_{28}$. This “delay” in the induction of DNA strand breakage is in accordance with our previous results and substantiates the hypothesis that contaminant interactions (especially PAH vs. metallic contaminants) might be important confounding factors for this biomarker (Costa et al., 2008a). In fact, antagonistic interactions between PAH and metals have already been described, occurring as a result of the inhibition by metals of the cytochrome P450 (CYP1A) --mediated PAH activation (e.g. Vakharia et al., 2001; Spink et al., 2002). Since PAHs have low solubility in water, cells promote detoxification by activating these compounds, converting them into the more soluble, highly reactive, PAH-quinones and the highly genotoxic PAH diol-epoxides, with production of ROS, a reaction that is normally catalyzed by the microsomal cytochrome complexes (Lemaire and Livingstone, 1997). Detoxification of PAHs is therefore a catabolic reaction that enhances mutagenicity by action of its activated (electrophilic) by-products that form adducts with the DNA molecules, promoting strand instability and eventual fragmentation, besides the direct action of ROS. Even though the liver is the primary vertebrate organ where xenobiotics are metabolized, ROS and other mutagenic substances are likely to spread via peripheral blood and affect blood cells and haematopoietic tissue. Conversely, inhibition of CYP1A activity might have reduced mutagenicity by liberating less genotoxic compounds into the blood stream. Chromosomal clastogenesis has also been linked to the formation of ROS (Soto-Reyes et al., 2005), which helps explaining the similar pattern of $VC_{TSB}$ and $VC_{ENA}$ in laboratory-tested fish and $VC_{ENA}$ in field-tested individuals. Organochlorines, like DDT and its metabolites, are also known to cause DNA strand fragmentation, as measured by the

SCGE (e.g. Yáñez et al., 2004). However, the low concentrations of organochlorines (translated into much lower *SQG-Q*s than for metals and PAHs) observed in the tested sediments make it difficult to determine their exact contribution to a potential synergistic or antagonistic effect on the observed TSB variation coefficients.

It should also be taken into consideration that there is evidence that many of the surveyed metals are known genotoxicants, even though the mechanisms of metal-induced mutagenesis remain somewhat unclear. Unlike activated PAHs, for instance, which may react directly with DNA, this effect is more indirect (e.g. by unbalancing oxidative metabolism and inhibiting DNA repair). It is the case, for instance of As, Cd, Co, Cr, Ni, and Pb (Hartwig, 1995) and both organic and inorganic species of Hg (Crespo-López et al., 2009). Since from the total *SQG-Q* for metals is higher for the sediment $C_1$ than for $C_2$ (0.79 and 0.58, respectively), this is yet another factor that allows a suspicion on the negative interactions between organic and metallic contaminants.

Field-tested fish, however, reveal a distinct pattern of induction of DNA strand damage. After fourteen days of exposure ($T_{14}$), test $C_1$ was the responsible for the greatest increment in TSB. At $T_{28}$, though, exposure to the reference sediment resulted in the highest increase in TSB whereas tests $C_1$ and $C_2$ remained unchanged. This increase in TSB as a result of exposure to the reference sediment is unlikely linked to sediment contamination. However, this effect is accompanied by a very significant increase in blood plasma lipid peroxides from $T_0$ to $T_{28}$ in field-exposed fish to the reference sediment. Since no alteration were observed regarding leukocyte counts, therefore excluding the action of natural parasites, it may be inferred that an unknown factor was responsible for the increase of $VC_{TBARS}$ and $VC_{TSB}$ in field-exposed fish to sediment R. One of the most plausible reasons is the oxidative stress originated by food deprivation. Site R is subjected to strong tidal currents and, when the animals were collected at $T_{28}$, sediment was observed to have been partially washed off from underneath the cage apparatus. Most animals were in fact found having empty digestive tracts, unlike fish allocated in stations $C_1$ and $C_2$, inside which remains of small gastropods and bivalves were commonly found. Senegalese soles are known to occupy sandy or muddy bottoms where they forage on small benthic invertebrates (Cabral, 2000). Starvation has been found to increase lipid peroxidation in teleosts (Pascual et al., 2003; Morales et al., 2004). Lipid peroxides result from the formation of ROS, for instance by depletion of anti-oxidant molecules like reduced glutathione (GSH) as a consequence of oxidative tissue damage (Robinson et al., 1997). It is therefore possible that fish tested on site R were deprived of food, e.g., by sediment washing-off, which is likely to have caused oxidative stress, resulting in an increase in both TBARS and TSB. It may also be one of the factors involved in the unexpected high mortality that occurred during this test. DNA oxidation has been described as a major mutagenic factor in organisms exposed to environmental xenobiotics (reviewed by Azqueta et al., 2009). It is also important to notice that changes in feed composition (affecting, e.g. availability of anti-oxidant elements like Se, dietary fat composition, etc.) might have had a significant role. A moderate but significant correlation was found between $VC_{TBARS}$ and $VC_{TSB}$ ($R = 0.26$, $p < 0.01$) for all tested fish, from which may be inferred that a factor affecting both markers (like oxidative stress)

may, at least partially, explain these results. Considering this information, it may also be argued that the lack of significant variation TBARS in laboratory-exposed fish may be, at least in part, explained by the combination of: reduction of oxidative stress by metal × organic contaminant interactions in tests $C_1$ and $C_2$; the reduced toxicity of the reference sediment and the controlled access to food. The lack of a clear relationship between environmental contamination and genotoxicity plus lipid peroxidation has already been described by other authors who proposed that unaccounted environmental and biological variables are important and require further research (e.g. Shaw et al., 2004). This relationship is also not clear at cellular and molecular levels and some authors have proposed that, independently of the oxidative offence, DNA strand breakage always depends on intrinsic factors such as cell division rates and DNA repair capability (Duthie and Collins, 1997; Hu et al., 1998).

Chromosomal clastogenesis, given by ENA, was apparently less susceptible to be affected by background variables than TSB, especially in the field assays where no significantly higher ENA frequencies in fish exposed to the reference sediment for 14 or 28 days, comparatively to $T_0$, were found. These results may derive from the different nature of the two types of mutagenicity: whereas TSB results from direct action of mutagens on the DNA chains, causing fragmentation, adducts and alkali-labile (chemically altered nucleotide) sites; ENA (like micronuclei) are considered to be linked to errors during the mitotic process, leading to DNA damage like chromosome fragmentation, failed segregation during nuclear division and isolation of whole chromosomes, and may thus magnify baseline DNA strand breakage (e.g. Schiffmann and de Boni, 1991; Neuparth et al., 2009). Although $VC_{ENA}$ and $VC_{TSB}$ were significantly correlated ($R = 0.45$, $p < 0.01$), a moderate negative correlation was found between $VC_{ENA}$ and $VC_{TBARS}$ ($R = -0.28$, $p < 0.01$), which is yet another evidence for the different mechanisms underlying the two types of mutagenesis. Considering that single-strand damage is repairable by DNA polymerases and ligases, clastogenesis might be considered a more severe type of mutagenesis. At some extent, as for DNA TSB, chromosomal clastogenesis has been found linked to oxidative stress (Cicchetti and Argentin, 2003) and therefore, an antagonist interaction between the different classes of contaminants may account for the similar pattern between $VC_{TSB}$ and $V_{ENA}$ within laboratory and field assays. It is also of notice that organochlorines (like PCBs and DDTs) are long known to be clastogenic (Hose et al., 1984, 1987). Still, the concentrations of the latest in the surveyed sediments were low, (highest *SQG-Q*s for PCBs and DDTs were 0.06 and 0.12, respectively, for sediment $C_2$) and were likely masked by metallic elements and PAHs, also known clastogenic compounds (e.g. Hose et al., 1984; Siu et al., 2004; Çavas et al., 2005).

Peripheral blood lymphocyte proliferation has already been described as an effect of exposure to PAHs, (Reynaud and Deschaux, 2005). On the contrary, Khan (2003) found reduced lymphocyte counts in feral flatfish collected from sites subjected to mixed classes of contaminants, especially PCBs and PAHs. Our study did not reveal any significant increase in leukocyte percentages between fish exposed to any of the sediments and $T_0$ animals. However, exposure to sediment $C_2$ (the most contaminated by PAHs), both in the laboratory and in the field, yielded a significant increase in

leukocyte counts (i.e. lymphocyte, since other immune cells were rare) after 28 days of exposure, comparatively to the exposure to the reference sediment. In the field assays, the same was observed for sediment $C_1$. These results suggest that exposure to the most contaminated sediments induced proliferation of peripheral lymphocytes; however, this comparison was only noticeable after a prolonged exposure. It should also be taken into consideration that $T_0$ fish essentially reflect the rearing conditions. Husbandry parameters such as population density may cause differences in the global immunological parameters of fish. Although the correlation between lymphocyte percentages, sediment contamination indices and genotoxicity biomarkers could indicate some potential value for ecological risk assessment, the mechanisms of leukocyte proliferation as a result of exposure to xenobiotics are not clear. Hooghe et al. (2000), for instance, suggested that xenobiotics may inhibit synthesis of cytokines, therefore impairing defence cell proliferation, which is not supported by the present findings. It may be inferred that, although possibly modulated by the presence of xenobiotics, caution is mandatory when considering this parameter as an indicator of exposure.

The present findings suggest that laboratory and field (*in situ*) assays may produce distinct results regarding the assessment of the genotoxic potential of sediment-bound contamination. Still, both yielded genotoxicity data globally consistent with sediment contamination. Overall, clastogenesis appears to better reflect the levels of sediment contamination in the field assays than total DNA strand breakage, a result that is in accordance with findings from other authors such as Wirzinger et al. (2007) with feral sticklebacks. Still, measuring DNA damage at the molecular level (as with the SCGE) is a more objective and sensitive method. Our results confirm the suitability of employing both bioassay methods to detect the genotoxic potential of sediment contaminants. We have also shown that oxidative stress biomarkers such as lipid peroxidation might be affected by factors external to contamination, although they greatly complement genotoxicity data. Cell type sorting, however, revealed highly variable data that may produce ambiguous or unclear results although leukocyte/lymphocyte counts may be of use when combined with other indicators and biomarkers perhaps as a general indicator of stress. This type of analysis might also be important, e.g., to infer the overall fish health status as a potential background variable in bioassays. Both types of bioassays (laboratory and *in situ*) are useful tools but each has its own advantages and handicaps. Laboratory assays might eliminate background variability of the measured effects and responses but may also unleash the full potential of contamination by favouring bioavailability, e.g. through sediment disturbance. Laboratory assays are especially useful when the main objective consists of assessing the net genotoxic potential (or its mechanisms) of contaminant mixtures, for instance as it might occur in the natural environment following sediment disturbance events (common in estuaries), such as dredgings, storms and strong tides or river flows. Still, it should also be taken into consideration that laboratory tests are likely to induce stress to animals, raising the background noise that will be cumulative with toxicity effects. Field studies may be more representative of the standard situation in a natural environment but are more susceptible to noise variables that induce variability, such as food availability and other environmental parameters that are very variable in estuaries such as salinity and

temperature fluctuations, complex hydrodynamics, etc., just to mention abiotic factors. Complementarily, our study showed that the use of sediment quality guidelines and contamination indices instead of net contaminant levels to compare the genotoxic potential of sediments with complex contaminant mixtures and that these indices provide a more realistic approach to interpreting data, especially from field assays, since laboratory tests are likely to magnify the effects of toxicity. The complexity of sediment contaminant mixtures and the effects of xenobiotic interactions need yet much research, especially when the toxic substances are not present in such high levels to provide distinguishable adverse effects on organisms.

To summarize, using fish peripheral blood to detect the genotoxic potential of sediment contamination is an expedite procedure capable of producing much information but integration with other blood parameters such as lipid peroxidation and cell sorting might be, at least, a useful guide to detect the effects of background variables. Although laboratory and field assays are useful tools, the choice for one of them should be based on a careful balance of advantages and disadvantages and always according to the real objectives of the study. Laboratory tests appear to be reliable tools than field assays for eliminating environmental variables, for being easier and cheaper to perform and by potentially exploring the full potential of sediment bound toxicants, thus providing a more conservative approach to biomonitoring procedures. However, field assays are likely to provide more realistic and ecologically relevant outcomes.

## Acknowledgements

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors would also like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and J. Raimundo, V. Branco, R. Cesário and P. Pousão (IPIMAR-INRB) for their support.

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## 4.2. Estuarine ecological risk based on hepatic histopathological indices from laboratory and *in situ* tested fish[†]


### Abstract

Juvenile Senegalese soles were exposed through 28-day laboratory and field (*in situ*) bioassays to sediments from three sites of the Sado Estuary (W Portugal): a reference and two contaminated by metallic and organic contaminants. Fish were surveyed for ten hepatic histopathological alterations divided by four distinct reaction patterns and integrated through the estimation of individual histopathological condition indices. Fish exposed to contaminated sediments sustained more damage, with especial respect to regressive changes like necrosis. However, differences were observed between laboratory- and field-exposed animals, with the latest, for instance, exhibiting more pronounced fatty degeneration and hepatocellular eosinophilic alteration. Also, some lesions in fish exposed to the reference sediment indicate that in both assays unaccounted variables produced experimental background noise, such as hyaline degeneration in laboratory-exposed fish. Still, the field assays yielded results that were found to better reflect the overall levels of contaminants and physico--chemical characteristics of the tested sediments.



### Key-words

*Solea senegalensis*; histopathology; weighted indices; contaminated sediments; estuary; bioassays


### 1. Introduction

The determination of the ecological risk of contaminated sediments has long been recognized as a key issue to assess the effects of anthropogenic pressure onto the natural environments, in this case, the release of pollutants to aquatic ecosystems. Aquatic sediments and, in particular, estuarine sediments, are complex media with respect to physical, chemical and biological characteristics that trap, store, modify and, under certain circumstances, release contaminants to the biota. For all these reasons, integrative, ‘‘holistic”, approaches have been attempted to evaluate sediment ecological risk, combining sediment geochemistry, biotic composition and diversity and, among other potential lines--of-evidence, the effects of sediment-bound contaminants to aquatic organisms (Chapman and Hollert,

[†] Costa et al. (2011). *Mar. Pollut. Bull.* **62**:55-65 (doi:10.1016/j.marpolbul.2010.09.009).

2006).

Due to its complex nature, the evaluation of sediment risk for biomonitoring, regulatory, or more baseline ecological and toxicological purposes (including the analysis of toxic effects to organisms), has been given particular attention. Although many studies focused on feral animals, bioassays are widely employed in ecotoxicological studies. Still, performing bioassays with natural sediments has many constraints, from the presence of contaminant mixtures (that may result in antagonistic or additive effects that mask the outcomes of individual contaminants) to the factors that affect bioavailability, as well as the often unpredictable environmental variables that cause experimental noise. The choice between laboratory and *in situ* (field) assays thus relies on the balance between the need to reduce the background noise of the experiment with the least compromise of ecologically relevant results. Few studies have, however, focused on the differences between laboratory and *in situ* assays and each type's assets and disadvantages (as, for instance, Vethaak et al., 1996; Hatch and Burton, 1999) and none were found comparing directly the histopathological results obtained from the two approaches even though Riba et al. (2005) found similar types and levels of lesions in Senegalese soles exposed to contaminated sediments from proximate areas in the laboratory and *in situ*, although the fish were not tested simultaneously. Still, some authors have discussed that the two types of bioassays are adequate for biomonitoring procedures in spite of differences in the toxicity effects to organisms (Hatch and Burton, 1999; Riba et al., 2005).

The employment of histopathological biomarkers to determine the effects of environmental contamination has been perceived as a highly relevant methodology since they reflect the true health state of the organism. With respect to aquatic environments, the fish liver has been considered one of the major targets of assessment due to its function in xenobiotic transformation, storage and, even, elimination, with the gills, kidneys, gonads and digestive tract being other common subjects (see Bernet et al., 1999; Wester et al., 2002; Au, 2004, for a review). Assessing hepatic histopathology in feral fish is long surveyed for biomonitoring and regulatory purposes. Among these studies, the survey of neoplasic or pre-neoplasic lesions in benthic fish, especially flatfish, is recurrent (e.g. Myers et al., 1998; Koehler, 2004; Lang et al., 2006).

If the importance of purely qualitative approaches to histopathology cannot be disregarded since it allows the detection and development of new potential biomarkers as well as the biological significance of the lesions and alterations (e.g. Köhler, 1990; Costa et al., 2010), semi-quantitative approaches are needed when it is intended to integrate biological data with environmental parameters through, e.g., multivariate statistics, in order to search for cause-effect relationships. Still, if these approaches are widespread concerning other classes of biomarkers, obtaining figures for histopathological traits is not yet a rule. This results mostly from (i) the difficulties of objectively identifying histological changes; (ii) the frequent lack of consensus between terminology and even identification of histopathological features; (iii) the many gaps that remain about the biological significance of the lesions or alterations to tissue and organs and (iv) the lack of important cause-effect information which, combined with the potential unspecific profile of histological changes, makes it

difficult to discriminate between the real effects or responses and experimental noise. It should be noted that although much information exists in the fields of biomedicine, histopathology data on fish is scarce and even scarcer on aquatic invertebrates, although such subject is out-of-scope of the present work.

Different attempts have been made to semi-quantify histopathological features in fish exposed to xenobiotics. Some authors developed tissue quality indices that are attributed to sites or treatments, e.g., by attributing an arbitrary degree of dissemination of one or more alterations within a given population (see for instance DelValls et al., 1998; Riba et al., 2005; Lang et al., 2006; Oliva et al., 2009). However, the development of individual indices is gaining interest. Among these, weighted indices are of especial relevance since they are based on the premise that the histological changes may not have the same impact (biological significance) to the animal. By attributing a numerical value to the relative importance (weight) of the alteration plus a dissemination factor, a histopathological condition indice can be obtained for each individual (Bernet et al., 1999; Costa et al., 2009b).

The Senegalese sole (*Solea senegalensis* Kaup, 1858; Pleuronectiformes: Soleidae) is a common flatfish in the Iberian Peninsula. It is a benthic fish that is often found in estuaries, preferring sandy-muddy bottoms where it feeds on small invertebrates (Cabral, 2000). The species is of ecological and economical importance in the study area of the present work, the Sado Estuary (Portugal, W Europe) and also an important aquaculture species in Southern Europe and the Mediterranean. Several ecotoxicological studies based on bioassays with the Senegalese sole have arisen in the past few years, taking advantage of the availability of the fish from aquaculture facilities and its benthic behaviour. These include laboratory exposure to waterborne or directly injected contaminants (Arellano et al., 1999; Prieto-Álamo et al., 2009; Oliva et al., 2009) and contaminated sediments (Riba et al., 2004, 2005; Salamanca et al., 2008; Costa et al., 2008, 2009a,b, 2010). The rising number of ecotoxicological studies with the species may indicate that *S. senegalensis* can achieve the potential in SW Europe that *Platichthys flesus* has been recognized with in the northwest for the environmental monitoring of marine and estuarine sediments. Still, much research is missing regarding the testing and validation of biomarkers and other indicators of aquatic pollution. Among the various responses and effects surveyed during these exposures, histopathological changes have also been evaluated (Arellano et al., 1999; Riba et al., 2005; Salamanca et al., 2008; Oliva et al., 2009; Costa et al., 2009b, 2010). Previous studies from our group showed that laboratory tests may enhance toxicity by increasing the bioavailability of the contaminants trapped in the sediments likely through the combination of fish- and sediment handling-driven resuspension and the sediments' physico--chemical properties like redox potential and organic matter, with consequences to the histopathological evaluation (Costa et al., 2009b, 2010). These findings led to the design of a new series of bioassays, performed simultaneously in the laboratory and in the field, using the same species and considering the same locations.

The present work aims to (1) identify histological lesions and alterations in the liver of *S. senegalensis* exposed to contaminated sediments and semi-quantify the results through

histopathological weighted indices; (2) compare the results between laboratory- and *in situ*-exposed animals to the same sediments in order to infer the advantages and handicaps of each type of assay as well as to determine potential confounding factors and (3) contrast the histopathological results to the sediments' characterization data to determine which histological biomarkers more effectively reflect the levels of contaminants, factors potentially affecting bioavailability and the effects of xenobiotic interactions.

## 2. Methods and materials

### *2.1. Study area*

The Sado Estuary (W Portugal) is a large basin of great ecological, social and economical importance. The estuary is historically subjected to many sorts of anthropogenic usage and alteration and includes a large city (Setúbal, with an important commercial harbour) and a dense agglomerate of heavy-industry (including chemical plants, a paper mill, a large thermoelectrical unit, shipyards and ore deployment facilities). It is also important for fisheries, tourism and aquaculture activities and a large portion of the estuary is classified as a natural reserve (Fig. 4.2.1). Three sites of the estuary were chosen according to previous research (Caeiro et al., 2005; Neuparth et al., 2005; Costa et al., 2009a). The reference site (R), located off the Tróia Peninsula, is the farthest from direct pollution sources (by more than three km). Sites $C_1$ and $C_2$ were considered the contaminated sites, although with different physico-chemical characteristics and levels of metallic and organic contaminants. They are located near Setúbal's harbour and off the city's heavy-industry belt, respectively.

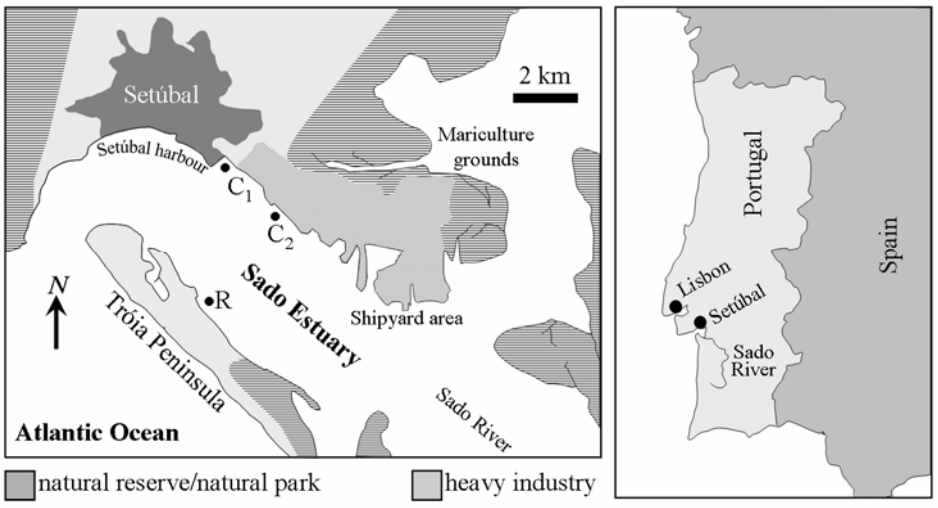


**Fig. 4.2.1**. Map of the Sado Estuary (W Portugal) with the location of the assay and sediment collection sites R (reference) and $C_1$ and $C_2$ (contaminated).

*2.2. Bioassays*

Sediments samples from the three sites (Fig. 4.2.1), for contaminant analyses and the laboratory assays, were collected with a Petite Ponar grab on May 2007. Juvenile laboratory hatched and reared Senegalese soles (standard length = 61.0 ± 8.4 mm; total wet weight = 3.1 ± 1.6 g), all from the same cohort, were used as test subjects. To simplify, exposures to sediments from sites R (reference) and $C_1$ and $C_2$ (contaminated) will throughout be referred to as tests R, $C_1$ and $C_2$.

The *in situ* (field) assays were set in the same areas where the sediments were collected. Submerged cages were placed over the bottom (ensuring direct contact with the sediment) by scuba diving (at 7-10 m depth). The cages consisted of 90 × 90 × 30 cm PVC plastic structures lined with a 5 mm plastic mesh. Each cage was divided in two equal-sized compartments (replicates), each allocating twenty randomly-selected animals. The laboratory assay was prepared according to previous research (Costa et al., 2009b). In brief: 2 L of freshly-collected sediments were placed in 15 L capacity white polyvinyl tanks with blunt edges to which was added 10 L of clean, 0.45 µm-filtered, seawater. Sediments (total surface ≈ 525 $cm^2$) were allowed to settle for 48 h before the beginning of the assay. The test tanks were equipped with a recirculation system and constant aeration, with water and air flows set to avoid sediment disturbance. The assays were performed in duplicate, with twenty randomly-selected animals being placed in each tank. A weekly 25% water change was done to maintain constancy of parameters with minimal removal of suspended particles and contaminants. Temperature was held constant as 18 ± 1 ºC and the photoperiod was set at 12:12 h light:dark. Water parameters were monitored weekly and were similar to the animals' rearing conditions: salinity = 32.1 ± 0.3, pH 8.0 ± 0.1, dissolved oxygen = 56.5 ± 0.2% and unionized ammonia ($NH_{3)}$ was restrained within 0.04 ± 0.02 $mg.L^{-1}$. Fish were fed daily with commercial pellets.

Field and laboratory assays were run simultaneously and had the duration of 28 days. Sampling was scheduled for days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$) of the experiment. At $T_{14}$ and $T_{28}$ three to five animals per replica were collected from each cage or tank and measured for total wet weight ($ww_t$) and standard length ($L_s$) before processing. Fish were then euthanized by cervical sectioning and liver portions were excised and prepared for subsequent histological analysis. $T_0$ fish consisted of ten animals collected from the rearing tanks.

*2.3. Sediment characterization*

Sediment redox potential (Eh) was measured immediately after collection using an Orion 20A apparatus equipped with a H3131 platinum electrode with an Ag/AgCl reference electrode. Sediment total organic matter (TOM) was inferred from organic carbon loss-on-ignition after sample heating at 500 ºC for 5 h. Fine fraction (FF), particle size < 63 µm, was determined by hydraulic sieving following disaggregation with pyrophosphate.

Sediment element contaminants, the non-metal selenium (Se); the metalloid arsenic (As) and

the metals cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn), were determined from dried sediment samples by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series equipment, after mineralization with acids (HCl, HNO3 and HF) in Teflon vials according to Caetano et al. (2007). Total mercury (Hg) was determined from dried sediment samples by atomic absorption spectrometry (AAS) according to Costley et al. (2000), after pyrolysis of the samples at 750 ºC in an oxygen atmosphere in a combustion tube attached to an AMA-254 mercury analyzer (Leco). The reference sediments MESS-2 and PACS-2 (National Research Council, Canada) and MAG-1 (US Geological Survey, USA) were analyzed by the same protocols to validate the procedure and the values were found within the certified range.

Sediment PAHs were determined from dried samples spiked with surrogate standards (from Supelco) by gas chromatography-mass spectrometry (GC-MS) as described by Martins et al. (2008), after Soxhlet-extraction with an acetone + hexane mixture, using a Finnigan GCQ system. Seventeen 3- to 6-ring PAHs were quantified. Organochlorines (18 PCB congeners and DDTs, namely *pp*'DDT plus the *pp*'DDD and *pp*'DDE metabolites) were quantified by GC with electron capture detection (ECD) from dried sediment samples following Soxhlet-extraction with n-hexane and fractioning in a chromatographic column according to Ferreira et al. (2003), using a Hewlett-Packard 6890 apparatus. Validation was achieved by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the obtained values were found within the certified range.

### *2.4. Histopathological analyses*

Liver portions were fixed in Bouin-Hollande's solution (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added till saturation) for 36 h at room temperature, washed in distilled water o/n, dehydrated in a progressive series of ethanol and embedded in paraffin (xylene was employed for intermediate impregnation). Sections (2-3 μm thick) were stained with haematoxylin and counterstained with alcoholic eosin (H&E). The procedure follows essentially Martoja and Martoja (1967). Other staining techniques were used to confirm the identification or highlight specific structures, namely: Sudan Black B for the histochemistry of protein-bound lipids in paraffin sections (prepared according to Bronner, 1975); Coomassie brilliant blue R250 (CBB) for the histochemical detection of protein (Fisher, 1968) and the Giemsa stain (in pH 4.8-5.8 phosphate-buffered saline) to aid identification of active Kupfer cells (after Kiernan, 2008). The slides were prepared in duplicate per sample (each containing eight to twelve sequential sections) and were mounted with DPX resin. All analyses were carried out using a DMLB model microscope (Leica Microsystems).

A semi-quantitative approach was enforced, based on the weighted histopathological condition indices proposed by Bernet et al. (1999). The estimation of the hepatic histopathological condition ($I_h$) indices is based on the concepts of: 1) each lesion or alteration's relative biological importance (weight) and 2) the score value, a numerical attribute that reflects the degree of dissemination of the

alteration within the surveyed organ. The indices are obtained for each individual and the histopathological alterations are divided by four reactions patterns, as defined by Bernet and co--workers: (1) circulatory disturbances; (2) inflammatory response; (3) regressive alterations and (4) progressive alterations. The $I_h$ indice for each fish is computed as:

$$I_h = \sum_{i=1}^{4} I_i \quad [1]$$

where $I_i$ is the condition indice for the $i^{th}$ reaction pattern (1-4), which is calculated by the formula:

$$I_i = \sum_{j=1}^{n} w_j a_j \quad [2]$$

where $j$ represents the $j^{th}$ lesion or alteration within each reaction pattern $i$ and $w_j$ and $a_j$ the weight and score, respectively, for the jth alteration. As defined by Bernet and co-workers, the score can attain the values of 0 (unaltered condition or unobserved lesion), 2 (infrequent occurrence); 4 (moderate occurrence) or 6 (severely disseminated/diffuse). A series of blind reviews of slides was employed to confirm the accuracy of observations.

*2.5. Statistical analyses*

Test statistics were performed on the individual $I_h$ and $I_i$ indices. Failure to meet the homogeneity of variances and/or the normality assumptions for parametric analysis of variance (given by the Levene and Kolmogoroff-Smirnoff tests, respectively), led to the employment of the non--parametric Mann-Whitney $U$ test to determine pairwise differences between $I_h$ values. Cluster analysis based on the 1-Pearson correlation $r$ statistic was used to investigate links between the $I_j$ values (weight × score) for the different histopathological traits observed. Discriminant analysis was used to determine the relative significance of each reaction pattern in the distinction between assay type and sampling time. The significance level was set at $\alpha = 0.05$ for all analyses. Statistics were obtained using Statistica (Statsoft Inc.).

## 3. Results

Distinct levels of aggregate mortality (after the 28 days of exposure) were observed between the laboratory and the field assays. In the laboratory experiment, exposure to sediment $C_2$ caused the highest (14 out of 40 individuals), followed by exposure to sediment R and $C_1$ (with 7 and 5 animals of 40, respectively). Unexpectedly, exposure to sediment R (the reference sediment) in the field was responsible for a comparatively high mortality (13/40) while only 5 of 40 fish were lost during both

tests $C_1$ and $C_2$.

*3.1. Sediment characterization*

The sediments from the three sites revealed distinct physicochemical properties and levels of contamination. The sediments from the reference site (site R) were found the least contaminated for both inorganic and organic contaminants, as well as the least anoxic and with the lowest percentage of fine grained particles and total organic matter (Table 4.2.1). Sediment from site $C_1$ was the most contaminated by metals, As and Se, whereas sediment from site $C_2$ was the most contaminated by organic xenobiotics, (PAHs and organochlorines), although the PAH levels were close to those of sediment $C_1$. Organochlorines were virtually absent from the reference sediment. The two contaminated sediments were found to be very anoxic and holding a high content of organic matter (highest for $C_1$). Four- and five-ring compounds represent ≈ 75-80% of all surveyed PAHs contamination in all sediments, the best represented being fluoranthene and pyrene with concentrations of 315.7 and 263.2 $ng.g^{-1}$ sediment dw in sediment $C_1$, respectively, and 345.2 and 286.3 $ng.g^{-1}$ sediment dw for $C_2$. Hexachlorinated PCBs were the most representative PCBs in all sediments, with the highest value being found in sediment $C_2$ (4.29 $ng.g^{-1}$ sediment dw).

*3.2. Liver histopathology*

Typically, fish collected at the beginning of the experiment ($T_0$) presented the normal hepatic architecture consistent with juveniles, showing regular hepatocytes, more or less polyedric in shape, with a clear cytoplasm, which should indicate good glycogen storage as previously described for the species (Costa et al., 2009b), and regular-sized nuclei with conspicuous nucleoli. At high-power magnifications, eu- and heterochromatin are clearly discernible. Many sinusoids could be observed branching out of larger blood vessels where a few blood cells (mostly erythrocytes) could be observed (Fig. 4.2.2A). The occurrence of lesions in the livers of $T_0$ fish was, in general, low.

Exposure to contaminated sediments, $C_1$ and $C_2$, globally caused the most pronounced alterations to the hepatic parenchyma. However, laboratory- and field-exposed fish depicted distinct patterns and levels of histopathological changes, most obvious in animals sampled after fourteen days of exposure, since at $T_{14}$ the livers of field-tested animals sustained the greater damage, especially in fish exposed to sediment $C_1$, contaminated by both element and organic xenobiotics. After 28 days, the exposure to all sediments, including to the reference sediment (R), was responsible for increased alterations to the hepatic parenchyma when compared to $T_0$ animals, even tough fish exposed to sediments $C_1$ and $C_2$ sustained greater damage in both type of assays, more notoriously for *in situ*--exposed animals.

Amongst the alterations most often observed in the livers of fish exposed to the two most contaminated sediments ($C_1$ and $C_2$); both in the laboratory and *in situ* (Fig. 4.2.2B-F), circulatory

disturbances and inflammatory response-related alterations were some of the most conspicuous. Haemorrhages were frequently observed around blood vessels, especially when blood-swollen vessels (leading to blood stasis) and proliferation of sinusoids indicated some degree of inflammatory response (Fig. 4.2.2B and 4.2.2E). Erythrocytes from ruptured vessels were often observed to intrude into foci of necrotic tissue spreading from the periportal area (‘‘piecemeal” necrosis). Necrotic foci were present in fish subjected to all treatments and $T_0$ fish. Although the extension and relative number of these foci were variable, fish exposed to sediments $C_1$ and $C_2$ sustained necrosis more diffusively, both in the laboratory and *in situ*, while in most animals exposed to the reference sediment (and $T_0$ fish), necrosis was either absent or constricted o small foci, usually around the periportal area. The most severe necrosis was observed in the livers of laboratory-tested fish exposed to sediment $C_1$ (contaminated by metallic and organic substances), collected at both $T_{14}$ and $T_{28}$, with necrotic areas being found disseminated throughout the entire organ and not just circumscribed to the periportal region. The most necrotic livers typically presented changes in the bile duct structure, exhibiting tubular structural regression and/or necrotic epithelia (Fig. 4.2.2B). Necrotic hepatocytes usually presented nuclear pleomorphisms, such as pyknosis or hypertrophy. Some evidence of apoptosis, revealed by changes in chromatin structure, was observed in the most damaged livers (Fig. 4.2.2C).

Altered hepatocytes (eosinophilic hepatocellular alteration) and lipidosis ("fat” degeneration) were found to be common alterations in fish subjected to all tests, with a variable degree of dissemination. Still, these alterations were more diffuse in field-exposed animals, including in fish tested in the reference site (R) for 28 days. No evidence for microvesicular fat degeneration (steatosis) was found. Altered hepatocytes typically presented more eosinophilic (acidophilic) cytoplasms’ (thus retaining much eosin, an acidic reddish pigment), accompanied by an alteration in shape and size, loosing their common polyedric outline and frequently presenting hypertrophy (Fig. 4.2.2C-E). Although eosinophilic hepatocellular alteration is considered a pre-neoplasic lesion (Koehler et al., 2004), no evidence was found for the presence of benign or malignant tumours in the livers of surveyed animals. Fat vacuoles could often be found inside altered hepatocytes, with the largest and most numerous being observed in field-exposed fish for 28 days. The most damaged livers frequently presented a combination of severe progressive and regressive changes (like necrosis and eosinophilic hepatocellular alteration, respectively). In these cases, Kupfer cells (liver-specialized macrophages) were often observed intruding into the damaged tissue (Fig. 4.2.2D and 4.2.2F), whereas melanomacrophages were more frequently observed in the periportal areas, occasionally forming dense centres (Fig. 4.2.2C).

The presence of various small intraplasmatic eosinophilic bodies (appearing as reddish circular or oval structures) inside hepatocytes was common in animals exposed to the two most contaminated sediments in both types of assays (hyaline degeneration). However, fish exposed in the laboratory to the reference sediment presented large, few or single, eosinophilic bodies that compressed the nucleus and cytoplasm against the plasmatic membrane (Fig. 4.2.2G). Laboratory--tested animals exposed to sediment R presented more diffuse hyaline degeneration (and larger

eosinophilic bodies) than $T_{14}$ fish. Still, the overall structural aspect of the hepatic parenchyma did not appear compromised, with little or no evidence for necrosis or eosinophilic hepatocellular alteration. These inclusions presented a clear halo and were strongly stained by eosin. Staining with Sudan Black yielded positive for protein-bound lipids inside eosinophilic bodies (Fig. 4.2.2H), as well as for total protein through Coomassie blue staining (Fig. 4.2.2I). Neither staining was observed to be homogenous, revealing the coexistence of different sorts of undetermined material inside the inclusions. Hyaline degeneration was not, nevertheless, observed in field-tested fish exposed to sediment R.

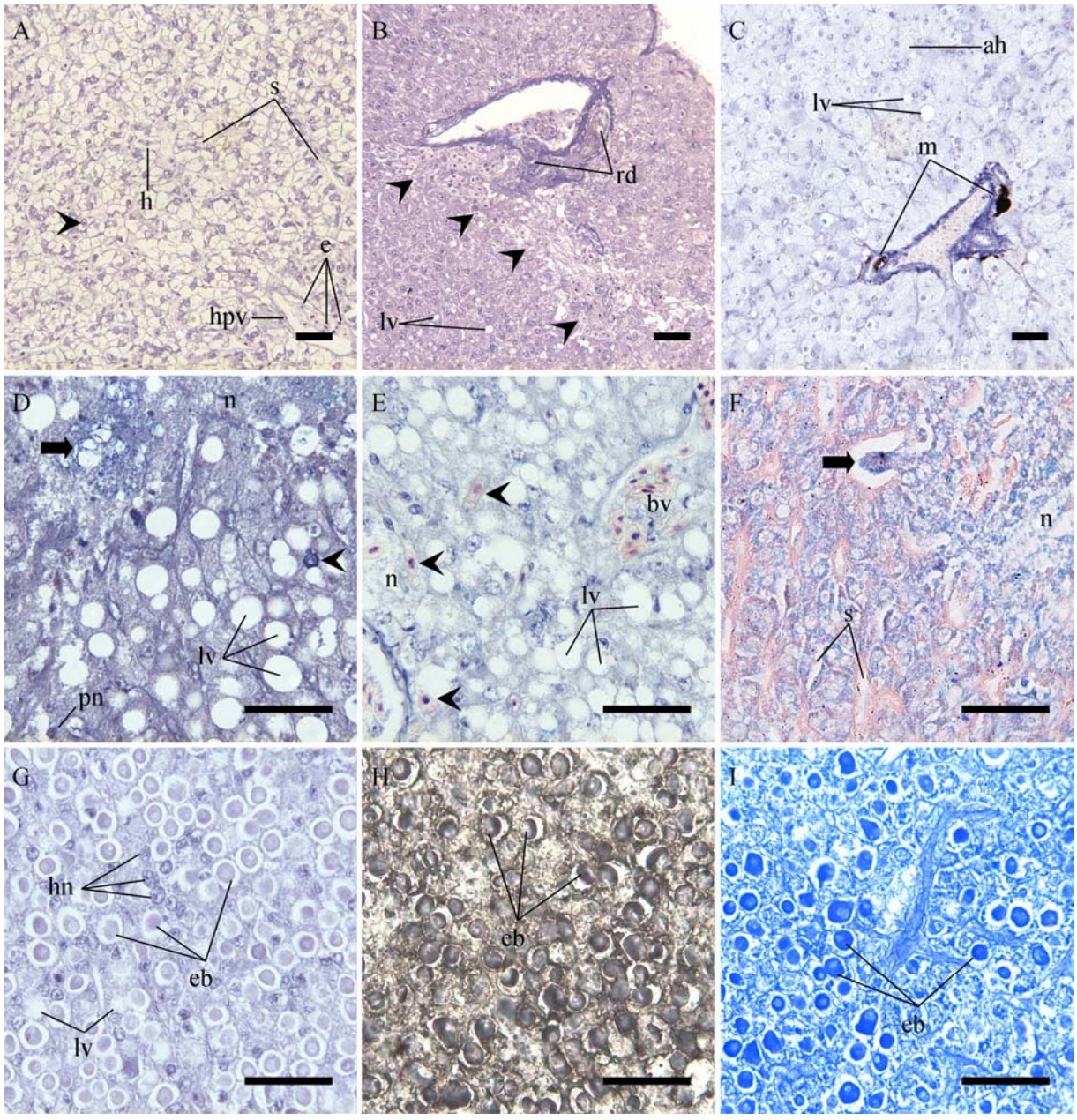


**Fig. 4.2.2**. Common histopathological lesions and alterations observed in the livers of laboratory- and *in situ*-exposed soles. Scale bar: 25 µm. A) overall aspect of the morphology of a normal juvenile liver (H&E). The hepatic parenchyma is composed of somewhat polyedric hepatocytes (h) with clear hepatocytes which should ▶

◀ indicate good glycogen storage (Simpson, 1992). Many sinusoids branch from larger blood vessels like branches of the hepatic portal vein (hpv) and contain sparse erythrocytes (e). Normal hepatocytes have constant-shaped nuclei with well-individualized eu- and heterochromatin and concentric nucleoli. B and C) hepatic parenchyma of a fish *in situ*-exposed to sediments from site $C_1$ (the most metal-contaminated), in the laboratory, for 28 days (H&E). B) a necrotic (arrowheads) area is spreading around a branch of the hepatic portal triad ("piecemeal" necrosis). The necrotic tissue is invaded by erythrocytes, indicating haemorrhage. The bile ducts of the triad also show signs of regression/necrosis (rd). Relatively small and sparse lipid vacuoles (lv) indicate moderate lipidosis. C) the liver of this individual presented massive hepatocellular alteration and modest lipidosis. ah) eosinophilic-altered hepatocyte; lv) lipid vacuole; m) melanomacrophages forming dense centres at a blood-swollen hepatic portal vein branch. D) detail of the liver of an *in-situ* exposed fish (site $C_2$, the most contaminated by organic xenobiotics) for 28-days (H&E). Nuclear pleomorphisms were commonly observed near or at necrotic areas (n), such as pyknosis (pn). A pleomorphic nucleus of undisclosed type (probably apoptotic) can also be observed (arrowhead) A Kupfer cell with many phagosomes can be observed nearby (arrow). (lv) indicates lipid vacuoles. E) Liver of a field-exposed fish in site $C_1$ for 14 days exhibiting many large fat vacuoles (lv) and an early-stage necrotic focus (n) around blood vessel with erythrocytes intruding into the damaged tissue (arrowheads). An adjacent blood vessel (bv) shows pronounced swelling caused by an increase in blood cells during inflammation (H&E). F) Detail of the liver of a field-exposed fish (site $C_1$) for 28 days were a Kupfer cell is observed intruding into a necrotic area (n) from an adjacent blood vessel (arrow). s) sinusoids (Giemsa stain). G-I) Eosinophilic bodies (hyaline degeneration) in the liver of animals exposed to the reference sediment for 28 days under laboratory conditions. G) H&E stain; H) Sudan Black B stain, signalling positive for protein-bound lipids inside the bodies and I) Coomassie brilliant blue stain for peptides with positive signal for peptide material inside eosinophilic bodies. eb) eosinophilic bodies; hn) hepatocyte nuclei; lv) lipid ("fat") vacuoles of common lipidosis appearing as empty-like structures in paraffin-embedded samples.

### *3.4. Hepatic histopathological condition indices*

The list of surveyed pathologies was determined from preliminary observations. Four reaction patterns were considered, each comprising one of several histopathological lesions or alterations to which was attributed its respective condition weight (Table 4.2.2). The weights ($w$) were attributed according to previous research (Bernet et al., 1999; Costa et al., 2009b). Circulatory disturbances and inflammatory response-related alterations (except Kupfer cell infiltration with $w = 2$) and lipidosis were attributed the lowest weights ($w = 1$) whereas necrosis was given the highest ($w = 3$). Nuclear pleomorphisms and bile duct structural changes received the intermediate value of $w = 2$, as well as hyaline degeneration and hepatocellular eosinophilic alteration, the latest being generally regarded as a pre-neoplasic alteration.

**Table 4.2.1**. Physico-chemical characterization of the sediments collected from the surveyed sites R (reference) and $C_1$ and $C_2$ (contaminated). Ranges indicate the quantification error.

| | | | Site | | |
|---|---|---|---|---|---|
| | | | R | $C_1$ | $C_2$ |
| | Eh (mV) | | -140 | -300 | -312 |
| | TOM (%) | | 23 | 96 | 76 |
| | FF (%) | | 2.3 | 10.2 | 7.2 |
| Element (μg.g$^{-1}$ sediment dw) | Non-metal | Se | 0.27 ± 0.01 | 1.21 ± 0.02 | 0.80 ± 0.02 |
| | Metalloid | As | 5.20 ± 0.10 | 23.98 ± 0.48 | 20.69 ± 0.41 |
| | Metal | Cd | 0.06 ± 0.00 | 0.26 ± 0.01 | 0.29 ± 0.01 |
| | | Co | 3.37 ± 0.07 | 13.94 ± 0.28 | 9.43 ± 0.19 |
| | | Cr | 18.14 ± 0.36 | 80.73 ± 1.61 | 51.70 ± 1.03 |
| | | Cu | 28.20 ± 0.56 | 172.72 ± 3.45 | 95.31 ± 1.91 |
| | | Hg | 0.11 ± 0.00 | 0.69 ± 0.01 | 0.71 ± 0.01 |
| | | Mn | 100.75 ± 2.01 | 464.34 ± 9.29 | 362.47 ± 7.25 |
| | | Ni | 7.31 ± 0.15 | 33.30 ± 0.67 | 20.49 ± 0.41 |
| | | Pb | 18.57 ± 0.37 | 55.19 ± 1.10 | 43.76 ± 0.88 |
| | | Zn | 72.29 ± 1.45 | 364.83 ± 7.30 | 269.31 ± 5.39 |
| Organic contaminant (ng.g$^{-1}$ sediment dw) | PAH | 3-ring | 15.29 ± 2.45 | 114.83 ± 18.37 | 100.70 ± 16.11 |
| | | 4-ring | 50.90 ± 8.14 | 701.19 ± 112.19 | 772.24 ± 123.56 |
| | | 5-ring | 26.65 ± 4.26 | 415.18 ± 66.43 | 423.92 ± 67.83 |
| | | 6-ring | 8.97 ± 1.43 | 133.99 ± 21.44 | 150.85 ± 24.14 |
| | *tPAH* | | *101.8 ± 16.3* | *1,365.2 ± 218.4* | *1,447.7 ± 231.6* |
| | PCB | Trichlorinated | 0.05 ± 0.01 | 2.33 ± 0.37 | 2.75 ± 0.44 |
| | | Tetrachlorinated | 0.03 ± 0.01 | 0.40 ± 0.06 | 1.08 ± 0.17 |
| | | Pentachlorinated | 0.03 ± 0.01 | 1.06 ± 0.17 | 1.61 ± 0.26 |
| | | Hexachlorinated | 0.41 ± 0.07 | 2.77 ± 0.44 | 4.29 ± 0.69 |
| | | Heptachlorinated | 0.27 ± 0.04 | 1.34 ± 0.21 | 2.24 ± 0.36 |
| | *tPCB* | | *0.80 ± 0.13* | *7.91 ± 1.26* | *11.97 ± 1.92* |
| | DDT | *pp*'DDD | < d.l. | 0.37 ± 0.01 | 0.71 ± 0.01 |
| | | *pp*'DDE | < d.l. | < d.l. | 0.59 ± 0.01 |
| | | *pp*'DDT | < d.l. | < d.l. | 1.22 ± 0.02 |
| | *tDDT* | | - | *0.37 ± 0.01* | *2.52 ± 0.05* |

[< d.l.], below detection limit; DDT, dichloro diphenyl trichloroethane; Eh, redox potential; FF, fine fraction (particle size < 63 μm); PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; TOM, total organic matter.

**Table 4.2.2**. Summary of the histopathological traits (biomarkers) assessed in the livers of tested *S. senegalensis* and respective weights.

| Reaction pattern | Histological alteration | Weight |
|---|---|---|
| *1. Circulatory disturbances* | Haemorrhage | *1* [a] |
| *2. Inflammatory response* | Profusion and dilation of blood vessels | *1* [b] |
| | Presence of melanomacrophages | *1* [b] |
| | Kupfer cell infiltration | *2* [a] |
| *3. Regressive* | Nuclear pleomorphisms | *2* [a] |
| | Hepatocyte necrosis | *3* [a] |
| | Bile duct regression/atrophy | *2* [a] |
| *4. Progressive* | Lipidosis | *1* [b] |
| | Intracellular eosinophilic bodies | *2* [b] |
| | Eosinophilic hepatocellular alteration | *2* [b] |

[a]weights according to Bernet et al. (1999)

[b]weights that follow Costa et al. (2009b)

With the exception of animals exposed to the reference sediment for 14 days, either in the laboratory or *in situ*, all tests caused an increase in the hepatic histopathological indice $I_h$ [1] relatively to $T_0$ animals, indicating that the fish were enduring lesions and alterations in the hepatic parenchyma throughout the assays (Fig. 4.2.3). Laboratory- and field-tested fish depicted distinct patterns of hepatic histopathological changes. Field- exposed animals to sediments $C_1$ and $C_2$ for fourteen days had $I_h$ values significantly higher than laboratory-exposed fish to the same sediments. Also, exposures to sediments $C_1$ and $C_2$ resulted in higher indices than exposure to the reference sediment for both types of assays at $T_{14}$ (Mann-Whitney *U*, $p < 0.05$). Still, no statistical differences were found between fish exposed to sediments $C_1$ and $C_2$ for either case. At $T_{28}$, however, $I_h$ for fish exposed to $C_1$, in the laboratory was significantly higher than R and $C_2$ tests ($p < 0.05$) but no significant differences where found between R and $C_2$ tests. Regarding the *in situ* assay, exposures to the most contaminated sediments revealed higher indices than exposure to the reference sediment ($p < 0.05$) without, however, being statistically different between each other.

Upon analysis of the indices for each individual reaction pattern [2] it was observed that there was a differential contribution of the reaction pattern to the global indice $I_h$ (Table 4.2.3). Regressive changes accounted for most variation of $C_1$- and $C_2$-tested fish relatively to animals exposed to the reference sediment. On the other hand, it was observed that lipidosis (a progressive alteration) was more frequent and severe in field-exposed fish. Circulatory changes were highly variable but the frequency and severity of intra-hepatic haemorrhages depicted a tendency to increase in animals exposed to the two most contaminated sediments in both laboratory and field tests. With respect to

inflammatory response, the differences between fish exposed to the reference sediment and those exposed to $C_1$ and $C_2$ were more notorious but laboratory-exposed fish only showed a significant increase in frequency and severity of these changes at $T_{28}$.

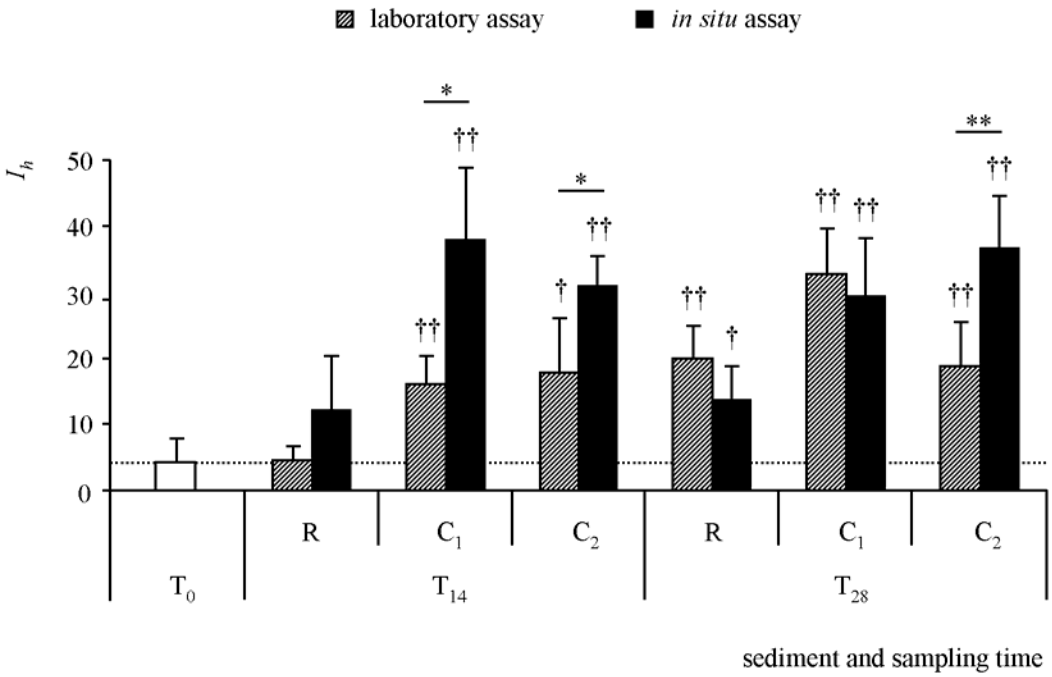


**Fig. 4.2.3**. Comparison of the average hepatic histopathological indice ($I_h$) between laboratory- and *in situ*-exposed fish to sediments from the reference (R) and contaminated ($C_1$ and $C_2$) sites at sampling times $T_0$, $T_{14}$ and $T_{28}$. * and ** mean significant differences between laboratory and *in situ*-exposed fish, $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney *U* test). † and †† indicate significant differences to basal $I_h$ at the beginning of the experiment ($T_0$ fish, dashed line), $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney *U* test). Error bars indicate 95 % confidence intervals.

Correlation-based cluster analysis on the individual indices for each histological change (weight × score) showed that some histopathological alterations were correlated (Fig. 4.2.4). Three unambiguous clusters were observed, the first comprising haemorrhage and blood vessel swelling; the second including Kupfer cell infiltration, nuclear pleomorphisms, hepatocyte necrosis and bile duct regression and the third joining lipidosis with hepatocellular alteration. Presence of melanomacrophages and eosinophilic bodies appeared as uncorrelated histopathological traits. Hepatocyte necrosis and nuclear pleomorphisms presented the strongest correlation.

From the discriminant analysis (Table 4.2.4) it was observed that at $T_{14}$, the reaction patterns that contributed the most to differentiate laboratory- and field-tested animals were inflammatory response and regressive changes, with the latest being the most significant reaction pattern at $T_{28}$. The reaction patterns of inflammation and progressive changes contributes the most to discriminate between laboratory-tested animals collected at $T_{14}$ from those collected at $T_{28}$. Conversely, no reaction patterns could significantly discriminate between field-tested fish collected at $T_{14}$ and $T_{28}$. Overall, only the discrimination between $T_{14}$ and $T_{28}$ for laboratory-exposed fish and between laboratory- and

field-tested fish sampled at $T_{14}$ were found to be significant (Wilk's $\lambda = 0.51$, $p < 0.01$ and Wilk's $\lambda = 0.56$, $p < 0.05$; respectively).

**Table 4.2.3**. Average hepatic histopathological condition indices for the four reaction patterns considered ($I_1$, $I_2$, $I_3$ and $I_4$), for all tests and sampling times. Ranges indicate 95% confidence intervals.

| **Sampling time** | $T_0$ | $T_{14}$ | | | | | $T_{28}$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Assay type | - | Laboratory | | | *In situ* | | Laboratory | | | *In situ* | | |
| Sediment | - | R | $C_1$ | $C_2$ | R | $C_1$ | $C_2$ | R | $C_1$ | $C_2$ | R | $C_1$ | $C_2$ |
| Reaction pattern | | | | | | | | | | | | | |
| **1. Circulatory disturbances** | | | | | | | | | | | | | |
| Haemorrhage | 0.5 ± 0.6 | n.o. | 0.6 ± 0.7 | 0.3 ± 0.7 | n.o. | 1.5 ± 1.9 | 0.4 ± 0.8 | n.o. | 1.0 ± 1.3 | 0.7 ± 1.3 | 0.3 ± 0.5 | 1.0 ± 1.0 | 0.5 ± 1.0 |
| $I_1$ | *0.5 ± 0.6* | *0* | *0.6 ± 0.7* | *0.3 ± 0.7* | *0* | *1.5 ± 1.9* | *0.4 ± 0.8* | *0* | *1.0 ± 1.3* | *0.7 ± 1.3* | *0.3 ± 0.5* | *1.0 ± 1.0* | *0.5 ± 1.0* |
| **2. Inflammatory response** | | | | | | | | | | | | | |
| Profusion and dilation of blood vessels | 0.5 ± 1.0 | n.o. | 0.6 ± 0.7 | 0.2 ± 0.3 | 0.5 ± 1.0 | 2.5 ± 2.5 | 0.8 ± 1.0 | n.o. | 2.0 ± 2.0 | 1.7 ± 1.2 | 1.5 ± 1.6 | 2.0 ± 1.7 | 2.0 ± 1.3 |
| Presence of melanomacrophages | 0.8 ± 1.0 | 0.4 ± 0.6 | 1.7 ± 1.3 | 1.0 ± 1.3 | 0.5 ± 1.0 | 1.0 ± 1.1 | n.o. | 1.0 ± 1.3 | 1.7 ± 1.6 | 2.7 ± 1.3 | 0.8 ± 0.7 | 1.3 ± 0.7 | 1.5 ± 1.0 |
| Kupfer cell infiltration | n.o. | n.o. | 0.6 ± 1.1 | 0.7 ± 1.3 | 2.0 ± 2.3 | 4.0 ± 3.2 | 5.6 ± 3.1 | n.o. | 3.3 ± 3.1 | 1.3 ± 1.7 | n.o. | 2.5 ± 2.5 | 5.0 ± 2.9 |
| $I_2$ | *1.3 ± 2.0* | *0.4 ± 0.6* | *2.9 ± 1.7* | *1.8 ± 1.5* | *3.0 ± 2.5* | *7.5 ± 5.2* | *6.4 ± 2.3* | *1.0 ± 1.3* | *7.0 ± 4.0* | *5.7 ± 1.9* | *2.3 ± 1.6* | *5.8 ± 2.6* | *8.5 ± 3.5* |
| **3. Regressive** | | | | | | | | | | | | | |
| Nuclear pleomorphisms | 0.5 ± 1.0 | n.o. | 4.0 ± 3.0 | 4.0 ± 3.5 | n.o. | 8.0 ± 3.2 | 6.0 ± 2.5 | 4.0 ± 2.9 | 6.7 ± 1.7 | 4.7 ± 2.4 | 0.5 ± 1.0 | 5.0 ± 2.0 | 6.0 ± 1.5 |
| Hepatocyte necrosis | 1.5 ± 1.9 | n.o. | 6.0 ± 2.6 | 5.0 ± 2.0 | 3.0 ± 3.4 | 12.0 ± 4.8 | 8.4 ± 2.9 | 3.0 ± 4.0 | 10.0 ± 2.5 | 3.0 ± 2.6 | 0.8 ± 1.5 | 7.5 ± 4.3 | 8.3 ± 3.1 |
| Bile duct regression | n.o. | n.o. | 1.1 ± 1.4 | 2.0 ± 1.8 | n.o. | 2.0 ± 2.3 | 0.8 ± 1.6 | n.o. | 4.7 ± 2.4 | n.o. | n.o. | 1.5 ± 1.4 | 3.0 ± 1.3 |
| $I_3$ | *2.0 ± 2.7* | *0* | *11.1 ± 4.3* | *11.0 ± 6.1* | *3.0 ± 3.4* | *22.0 ± 5.3* | *15.2 ± 3.4* | *7.0 ± 5.5* | *21.3 ± 5.1* | *7.7 ± 4.7* | *1.3 ± 2.4* | *14.0 ± 6.3* | *17.3 ± 5.4* |
| **4. Progressive** | | | | | | | | | | | | | |
| Lipidosis | 0.5 ± 0.6 | 2.2 ± 1.0 | 0.9 ± 0.8 | 1.3 ± 1.7 | 5.0 ± 1.1 | 5.0 ± 1.1 | 5.6 ± 0.8 | 2.7 ± 1.3 | 1.3 ± 1.7 | 0.7 ± 0.8 | 3.8 ± 1.9 | 3.8 ± 1.2 | 5.0 ± 1.0 |
| Intracellular eosinophilic bodies | n.o. | 1.8 ± 1.9 | 0.6 ± 1.1 | 2.0 ± 2.7 | n.o. | n.o. | n.o. | 9.3 ± 2.6 | 0.7 ± 1.3 | 0.7 ± 1.3 | n.o. | 0.5 ± 1.0 | 0.5 ± 1.0 |
| Eosinophilic hepatocellular alteration | n.o. | n.o. | n.o. | 1.3 ± 1.7 | 1.0 ± 2.0 | 2.0 ± 3.9 | 3.2 ± 3.8 | n.o. | 1.3 ± 2.6 | 3.3 ± 4.3 | 6.0 ± 3.0 | 4.5 ± 2.7 | 5.0 ± 2.9 |
| $I_4$ | *0.5 ± 0.6* | *4.0 ± 2.4* | *1.4 ± 1.1* | *4.7 ± 3.7* | *6.0 ± 2.8* | *7.0 ± 3.4* | *8.8 ± 3.4* | *12.0 ± 2.7* | *3.3 ± 2.4* | *4.7 ± 4.1* | *9.8 ± 3.4* | *8.8 ± 3.0* | *10.5 ± 2.2* |

n.o. - alteration/lesion not observed

**Table 4.2.4**. Discriminant analysis results taking assay type and sampling time as grouping variables (factors). Lowest Wilk's λ statistic was employed to assess best model. The most significant variables within a model were determined by *F*-tests following sequential addition of variables. The dependent variable is the global histopathological condition indice ($I_h$) per each individual.

| | | | Variables | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Model | $I_1$ | | $I_2$ | | $I_3$ | | $I_4$ | |
| | | Wilk's λ | Wilk's λ | *p* to remove | Wilk's λ | *p* to remove | Wilk's λ | *p* to remove | Wilk's λ | *p* to remove |
| Factors to discriminate | Case | | | | | | | | | |
| Assay type: | $T_{14}$ | 0.56 * | 0.57 | 0.68 | 0.67 | 0.02 | 0.56 | 0.73 | 0.64 | 0.05 |
| laboratory × *in situ* | $T_{28}$ | 0.86 | 0.86 | 0.93 | 0.90 | 0.18 | 0.87 | 0.53 | 0.97 | 0.04 |
| Sampling time: | Laboratory | 0.51 ** | 0.52 | 0.60 | 0.74 | 0.00 | 0.52 | 0.37 | 0.79 | 0.00 |
| $T_{14} \times T_{28}$ | *In situ* | 0.90 | 0.91 | 0.60 | 0.90 | 0.96 | 0.91 | 0.45 | 0.97 | 0.12 |

*,** - significance level for the model, $p < 0.05$ or $p < 0.01$, respectively (*F*-test)

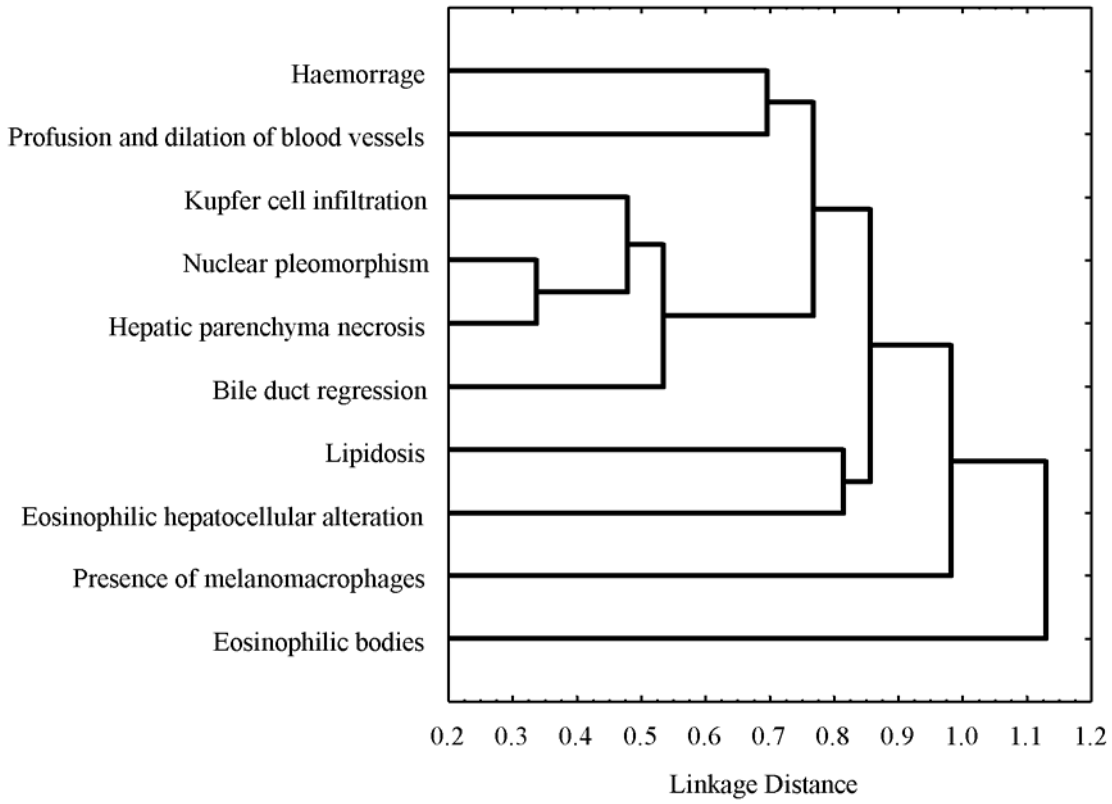


**Fig. 4.2.4**. Cluster analysis for all prospected histopathological biomarkers. Distances are based on the 1-Pearson correlation statistic *r* between condition indices. Amalgamation was achieved through unweighted pair-group averages.

## 4. Discussion and conclusions

The present findings indicate that laboratory and field bioassays may yield histopathological observations that are consistent with the overall contamination levels of estuarine sediments. However, it has also been demonstrated that there are significant differences between the two types of exposure regarding not only the increase in the global hepatic condition indice $I_h$ comparatively to the reference exposure but also the relative importance of each surveyed reaction pattern. Differences between laboratory-tested and field-collected or exposed organisms have already been reported by other authors and recognized as an important constraint when identifying the real toxicopathic effects of xenobiotics (see, for instance, Vethaak et al., 1996; Hatch and Burton, 1999). These differences are likely caused by (i) assay-induced factors that enhanced contaminant bioavailability in the laboratory assays and (ii) unmanageable environmental variables that affected field-tested animals such as access to food.

With the exception of fish exposed to sediment $C_2$, in the laboratory, for 28 days, $I_h$ levels

were significantly higher in fish exposed to the two contaminated sediments, $C_1$ and $C_2$, when compared to the reference test and $T_0$ individuals. However, no differences were found between $C_1$ and $C_2$ exposures in the field assays and, as to the laboratory experiment, only at $T_{28}$ fish exposed to $C_2$ revealed greater histopathological changes comparing to $C_1$. This can be partially explained by the fact that, unexpectedly, the levels of metallic and organic contaminants in the two most contaminated sediments, $C_1$ and $C_2$, were found more similar than in previous research (with sediments collected at proximate locations), mostly due to an increase in PAH contamination in sediment $C_1$ and metals in $C_2$ (see Costa et al., 2009a). Nevertheless, with respect to the levels of toxicants, the sediments tested during the present work can be considered clean (R) to moderately contaminated ($C_1$ and $C_2$). Another factor that contributed to the dilution of the differences between tests $C_1$ and $C_2$ concerns the interaction between metallic and organic xenobiotics, an issue reported in previous research (Costa et al., 2009b), especially the antagonistic effects between metals and PAHs.

Polycyclic aromatic hydrocarbons are insoluble compounds whose elimination depends on the inactivation by the cytochrome P450 (CYP) monooxygenase complex, producing more soluble compounds like the highly genotoxic diol-epoxides and reactive oxygen species (ROS) as by-products (see Miller and Ramos, 2001 for a review). Besides the importance of the non-metallic micronutrient selenium to anti-oxidant defences (Martínez-Álvares et al., 2005), best represented in sediment $C_1$, metals may have reduced immediate PAH toxicity by impairing CYP induction and activity (e.g. Vakharia et al., 2001), causing a ‘‘delay” in the severity of histopathological damage in fish exposed to the most contaminated sediment ($C_1$). If this ‘‘delay” in the effects has, indeed, occurred, that might contribute to explain the very significant increase in the $I_h$ values, for fish exposed in the laboratory to the sediment $C_1$, between $T_{14}$ and $T_{28}$, (inclusively, very significantly increasing over $C_2$-tested fish), which was not observed in field tests. It should be noted that the existence of severe regressive lesions account the most for the higher $I_h$ of $C_1$-exposed fish for 28 days compared to test $C_2$, especially necrosis and bile duct damage. This may indicate that antagonistic effects between the different types of contaminants may mask the true toxicopathic effects at early stages of exposure without, however, contributing to a real attenuation. Regarding *in situ* exposure, if the physico-chemical characteristics of the sediment did not so drastically change, it can be inferred that contaminants may have remained trapped in the sediments meaning that the full toxicological potential was not triggered. As a consequence, this antagonistic effect between element and organic contaminants was not so pronounced, leading only to a more marginal increase in the global histopathological indice $I_h$ of $C_1$--exposed fish relatively to $C_2$. To this may be added the fact that the levels of metallic and organic contaminants in both sediments were somewhat more resembled than expected and that the two classes of pollutants have different behaviours regarding their release from sediments. Organic compounds, especially PAHs, which are hydrophobic and essentially adsorbed to the sediment’s fine particles and organic matter, are more difficultly released to the water column under stable conditions of the upper layers of sediment but disturbance events combined with low Eh may favour their release, increasing bioavailability (refer to Eggleton and Thomas, 2004, for a review). It is likely that

laboratory sediments favoured contaminant bioavailability in tests $C_1$ and $C_2$ through a combination of sediment handling and animal-driven resuspension with high anoxia, TOM and FF. This enhanced bioavailability may have amplified antagonistic interaction effects in laboratory-tested fish (Costa et al., 2009b). Although this interaction might also have affected fish exposed to sediment $C_2$, its effects were likely more pronounced in fish exposed to sediment $C_1$, with higher levels of metals.

The histopathological changes observed appear to be unspecific to a particular set of contaminants, regardless of reaction pattern, but they reflect the global aspects of sediment contamination. Inflammation and circulatory disturbances (reaction patterns 1 and 2, respectively) were observed to be very variable, but follow the overall histopathological condition of tested fish. Interestingly, Kupfer cell intrusion (more notorious in field-exposed fish) was found to be well correlated to more severe alterations such has hepatocyte necrosis. Besides phagocytosis, Kupfer cells are known to have a role in intercellular communication in the presence of a particular set of contaminants, regardless of reaction pattern, but they reflect the global aspects of sediment contamination. Inflammation and circulatory disturbances (reaction patterns 1 and 2, respectively) were observed to be very variable, but follow the overall histopathological condition of tested fish. Interestingly, Kupfer cell intrusion (more notorious in field-exposed fish) was found to be well correlated to more severe alterations such has hepatocyte necrosis. Besides phagocytosis, Kupfer cells are known to have a role in intercellular communication in the presence of a xenobiotic challenge, e.g., by releasing tumour necrosis factor, TNF (Milosevic et al., 1999), which contributes to explain the link between infiltration and strong parenchyma damage. The activation of this liver-specific response has been found, for instance, to be triggered by metals and an organochlorine pesticide, lindane, with evidence for an agonist interaction (Junge et al., 2001), which contributes to the assumption that Kupfer cell infiltration is nonspecific to xenobiotic types. Intrusion of melanomacrophages, on the other hand, was very variable and was not found clearly correlated with any other histological change, although a link was observed between melanomacrophage intrusion and agglomeration with the sediment contamination levels. Interestingly, Miranda et al. (2008) found histological evidence that supports the occurrence of immunosuppression in feral teleosts exposed to environmental organochlorines, leading to reduced presence of melanomacrophages. Conversely, some authors found the presence of dense melanomacrophage aggregates in fish liver and other organs a good biomarker of general exposure to environmental contaminants (e.g. Oliveira Ribeiro et al., 2005). It is probable that intrusion of these defence cells is variable and modulated by factors other than the degree of damage in the liver.

Hepatocellular eosinophilic alteration and lipidosis were more disseminated in field-tested fish. Although the occurrence of both progressive changes is well documented in literature, the exact causes and consequences of both are not yet fully understood. Although eosinophilic or basophilic hepatocellular alterations are regarded as pre-neoplasic, these histopathological traits are considered to be reversible. Its exact biological consequence is unclear but Koehler and co-workers (2004); for instance, found that the metabolic activity is upregulated in pre-neoplasic eosinophilic hepatocytes in

feral flounders from PCB-contaminated sites. With respect to lipidosis, although some authors suggested that fact vacuolation is a response mechanism to store liposoluble xenobiotics (such as PAHs and organochlorines) or their metabolites (e.g. Köhler, 1990; Biagianti-Risbourg et al., 1995) this feature is more commonly regarded as an unspecific alteration with multiple potential causes. Interestingly, our previous work with *S. senegalensis* exposed to sediments from the proximate locations already reported a correlation between the presence of hepatocellular alteration and lipidosis and a link between these alterations and sediment contamination (Costa et al., 2009b). Similarly, the level of dissemination of both alterations is better linked to contamination in the laboratory assay than in the field experiment (where even fish exposed to the reference sediment presented diffuse forms of both), revealing that these changes may be triggered by undisclosed environmental factors. Fatty livers are common in aquaculture-brooded fish and may depend on diet. Tucker et al. (1997) found no short--term adverse effects of this condition on the livers of farmed fish, however, other authors discussed that fatty livers may have their energy production and anti-oxidant responses impaired (Vendemiale et al., 2001). Morales et al. (2004) found that, although reversibly, food deprivation causes oxidative stress and increases lipid peroxidation in teleost livers. It is possible that environmental factors such as access to food are, at least partially, responsible for the dissemination of fatty livers in field-exposed fish to the reference sediment, which might also account for some of the observed mortality. In fact, whereas in fish tested in sediments $C_1$ and $C_2$ the remains of small bivalves and gastropods were found in the digestive tracts, fish tested in sediment R frequently presented no signs of recent meals. This may be linked to the fact that the reference area has higher hydrodynamics, causing the upper layer of sediment to be more labile thus making access to preys more difficult. The animals may thus have had their fat metabolism altered and, as a consequence, be more prone to acquire hepatocellular dysfunctions as a result of increased oxidative stress and weakened anti-oxidative responses (see Sánchez-Pérez et al., 2005). Overall, the combined geophysical characteristics of the reference area are likely to have had a considerable, yet unmanageable, effect of the health status of the animals, whether by impairing access to food or some sort of physical stress. Fish exposed to the two contaminated sediments, on the other hand, may have suffered from lipidosis and eosinophilic alteration mostly as a result of exposure to xenobiotics, from metals (Arellano et al., 1999; Shaw and Handy, 2006; Giari et al., 2007), organic toxicants such as organochlorines (Miranda et al., 2008), or sediment-based mixtures (Oliveira Ribeiro et al., 2005; Costa et al., 2009b). Lipidosis has even been found a persistent trait in fish recovering from Cu exposure (Shaw and Handy, 2006), which may premeditate caution when interpreting this biomarker.

Intraplasmatic eosinophilic bodies were observed to contain protein and protein-bound lipids, which is in accordance with the observations by Koller (1973), who first observed tat these inclusions are constituted by amorphous peptidic and lipidic material, consistent with enlarged lysosomes. The same author ruled out a pathogenic origin to hyaline degeneration. The absence of defence cells other than macrophages (namely lymphocytes) indicates that this alteration was unlikely caused by infectious agents. Unlike previous research, when eosinophilic bodies (occasionally termed hyaline

degeneration) were found in conjunction with hepatic necrosis and linked to the exposure to organic xenobiotics (Costa et al., 2009b), these inclusions were found to be unrelated to severe regressive changes. Accordingly, other authors reported the existence of multiple small eosinophilic inclusions in the liver of fish exposed to metals without, however, coexistence with severe structural damage (Van Dyk et al., 2007). Similarly, biomedical research has reported the existence of these inclusions in human neoplasic livers but no direct link with patient survival was found (Chedid et al., 1999). It is possible that hyaline degeneration is an unspecific alteration with multiple, yet unknown, causes, as suggested by previous work from our group (Costa et al., 2009b). The presence of multiple small eosinophilic structures inside hepatocytes of fish exposed to the contaminated sediments resemble the Mallory-Denk bodies described for mammals, which are known to have a very ubiquitous origin (see Strnad et al., 2008). Although hyaline degeneration can be found together with other severe lesions, it appears that its actual significance may be masked by multiple confounding factors which implicates that further research is yet needed before the presence of eosinophilic bodies in hepatocytes can be regarded as a potential histopathological biomarker of environmental contamination on its own.

Histopathological biomarkers proved to be solid tools to monitor sediment-bound contaminants in estuaries if integrated through a semi-quantitative arrangement of condition indices that take into consideration not just the degree of dissemination but also the biological importance (“weight”) of the lesion or alteration. The individual indices were proved to be highly advantageous when analysing the data through, e.g., multivariate statistics, yet another asset demonstrated elsewhere (Costa et al., 2009b). Provided that the proper weights are attributed and that possible confounding factors are taken into consideration, it appears to be advantageous to assess as many histopathological traits possible in order to cope with inter-individual variation, the unspecificity of lesions to a particular class of contaminants and the fact that some reaction patterns may be better indicators of the global health status of the fish than others as did regressive changes in the present study. It is also important to notice that histopathological lesions are likely to appear, in laboratory and field bioassays, even in ‘‘control” or ‘‘reference” organisms, so a comparative approach is compulsory, as well as the choice of an adequate reference. Both laboratory and field assays could provide results that could correlate to the global sediment contamination, a result that can relate to previous findings when histopathology was employed in biomonitoring procedures (Riba et al., 2005). These results indicate that semi-quantitative histopathological analyses in fish are an adequate approach to take part of ecological risk assessment strategies, regardless of the assay methodology. However, field assays provided clearer comparisons between contaminated and clean sites even though unknown environmental factors caused some degree of experimental noise. Also, the integration of multiple histopathological biomarkers or potential biomarkers into combined indices that consider both the biological importance of the change as well as its degree of dissemination allows surpassing some of the inter-individual variation and assay or environment-induced variability, in spite of the non--specificity of lesions to a toxicant or class of toxicants.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). For their support, the authors would like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA) and J. Raimundo, V. Branco, R. Cesário and P. Pousão (IPIMAR-INRB).

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## 4.3. Evaluation of stress-related gene transcription profiles in flatfish exposed to contaminated estuarine sediments[†]


### Abstract

Transcriptomics is a novel tool gaining ground in ecological risk assessment (ERA) for being a high power approach for both predictive and mechanistic toxicology. Even though benthic fish are of recognized value for the monitoring of aquatic sediments, there still remains the need to improve gene sequencing of these species and to design and validate gene transcription arrays as potential biomarkers of exposure, especially when complicated geochemical matrices and mixtures of xenobiotics are at stake, as in estuarine sediments. The transcription of a battery of contaminant response-related genes was investigated in juvenile Senegalese soles (*Solea senegalensis* Kaup, 1858), exposed to sediments from three distinct sites (a reference plus two contaminated) of a Portuguese estuary (the Sado, W Portugal) through 28-day laboratory and field (*in situ*) bioassays. The transcription of cytochrome P450 1A (CYP1A), metallothionein 1 (MT1), glutathione peroxidase (GPx), catalase (CAT) and caspase 3 (CASP3) genes; all identified and partially characterized at the sequence level for the first time in this species; plus the 90kDa heat-shock protein alpha (HSP90AA), were surveyed by real-time RT-PCR, which was complemented by assessing apoptosis through the TUNEL reaction. After 14 days of exposure, the relative number of mRNA copies of the surveyed genes presented little variation or significantly decreased in animals exposed to the contaminated sediments, indicating the existence of a disturbance stress phase during which the fish were unable to respond to insult. After 28 days of exposure, all genes' transcription responded to contamination but laboratory and field assays revealed distinct patterns of regulation, likely linked to increased bioavailability and to confounding variables, respectively. Although sediments revealed a combination of organic and inorganic toxicants, the relative transcription of the CYP1A gene was consistently correlated to organic contaminants. Metallothionein regulation, however, was found correlated to metallic contamination in the laboratory and to organic in the field assays. The transcription of oxidative stress-related genes can be a good indicator of general stress but caution is mandatory when interpreting the results since transcription regulation may be influenced by multiple factors. As for MT1, HSP90 upregulation has potential to be a good indicator for total contamination, as well as the CASP3 gene, even though hepatocyte apoptosis depicted values inconsistent with sediment contamination, showing that programmed cell death did not directly depend on caspase transcription alone.


[†] Costa et al. (*submitted*).

**Key-Words**

Toxicogenomics; Transcriptomics; *Solea senegalensis*; Quantitative real-time RT-PCR; Apoptosis; Ecological risk assessment

## 1. Introduction

Although a great deal of literature reports on the effects and responses of aquatic organisms triggered by the exposure to classical model xenobiotics or novel chemical stressors, surveying the effects and responses to sediment contaminants remains yet a challenge to environmental toxicologists. Aquatic sediments function as a complex geochemical trap that store, modify and under certain circumstances release contaminants back to the water column where, having reached a given threshold that depends on factors such as chemical speciation and biological species of the target organism, become pollutants if indeed toxic effects are exerted (Chapman, 2007). Besides all the physical and chemical characteristics that determine contaminant bioavailability (therefore modulating its toxicity), aquatic sediments are often characterized by the storage of multiple xenobiotics of distinct classes. The combination of diverse pollutants (e.g. metals, polycyclic aromatic hydrocarbons, pesticides, etc.) relates to the different sources of natural (as for some metals and metalloids) and anthropogenic contamination that marked coastal areas, especially estuaries, since the dawn of the Industrial Revolution. The co-exposure to multiple toxicants poses another challenge to toxicologists since biomarker responses often yield results that are inconsistent with the levels on sediment contamination or contradictory to published information regarding their specificity towards a single substance or a given class of xenobiotics (see, for example, Costa et al., 2009a, 2009b). Regarding this issue, much research is still missing not only to understand the complex biological mechanisms caused by exposure to contaminant mixtures but also to search, develop and validate relevant biomarkers.

The search for adequate biomarkers in fish for ecological risk assessment (ERA) is leading towards more integrative, holistic, approaches in order to either find indicators for mixtures of contaminants, or those that retain some degree of specificity even in presence of multiple xenobiotics (for predictive toxicology), or even to survey the complex machinery of response and defence against chemical insult (mechanistic toxicology). The novel “omic” approaches towards environmental toxicology began over a decade ago and comprises proteomics, metabolomics and transcriptomics, the latest often referred to as toxicogenomics in the field of research. Toxicogenomics thus results from the fusion between conventional toxicology and functional genomics, based on advanced molecular biology tools (see Gatzidou et al., 2007 for a thorough review on this subject). Although post--transcriptional mechanisms (influenced by multiple endogenous and exogenous variables) affect mRNA translation into a polypeptide and its maturation into a fully functional protein, transcription is a crucial point in the control of gene expression. To survey gene expression at the transcript level

several methodologies have emerged, including the recent high throughput sequencing approaches directed at whole transcriptomes like the cDNA microarray technology. Although screening of changes in gene transcription through cDNA microarrays is a popular technology for providing information at genome-wide level, its application is costly and restricted to taxa where considerable genomic or expressed sequence tag (EST) information is available. On the other hand, expression analysis of specific genes by reverse transcription polymerase chain reaction (RT-PCR) -related techniques, namely real-time RT-PCR, can be applied to any species as long as it is possible to design primers for amplification of target genes. In fact, the search for candidate genes in non-model species has been developed by designing primers from consensus sequences taking advantage of homologous gene sequences or ESTs previously deposited in open databases such as GenBank (see Snell et al., 2003). The analysis of gene transcription by qRT-PCR is a sensitive and reliable technique that directly surveys the amount of steady-state level of mRNA which reflects the production and stability of transcripts. In addition, this approach has been supplying open-access databases with the much needed EST information on many species as well as crucial insights on the mechanistics of toxicity.

For a growing number of eco- or environmental toxicologists, determining the transcription regulation patterns of specific genes related to contamination by chemical stressors, especially those concerning classic biomarkers such as CYP1A (cytochrome P450 1A) and MTs (metallothioneins) has been widely employed in the past decade as an alternative to traditional methods of protein determination. Many such studies considered piscine test species in a great variety of scenarios, from Antarctic waters (Miller et al., 1999) to estuarine and freshwater species, feral or subjected to laboratory and field bioassays (e.g. Wirgin and Waldman, 1998; Brammel and Wigginton, 2010). The success of this approach to study the mechanisms behind exposure to complex mixtures of xenobiotics has already been demonstrated (e.g. Filby et al., 2007, for the estrogenic effects of effluents). Amongst such research lie sediment risk assessment studies through fish-based bioassays and, within these, assays performed with flatfish (Teleostei: Pleuronectiformes). Flatfish species are gaining growing interest in sediment risk assessment for aquatic ecosystems due to their benthic nature, ecological importance and economical value. Still, many gaps persist in the knowledge of their physiology, ecology and genomics. For such reason, efforts have been put in hand to fill in these gaps, including "omic" approaches, among which transcriptomics earned a particular importance (Cerdà et al., 2010).

The Senegalese sole (*Solea senegalensis* Kaup, 1858), is a common species in the Iberian Peninsula, with a considerable economical value, including for aquaculture. The species occupies sandy-muddy bottoms of coastal areas and estuaries, particularly, important for breeding and nursing (Cabral, 2000). A considerable number of research reports sprung out in the past using this species as test subject, some of them aiming at ERA and toxicity testing, most for aquatic ecology and, very importantly, aquaculture. Among these studies there are surveys based on more traditional biomarker approaches (for instance Costa et al., 2009a, 2009b; Chairi et al., 2010; Kalman et al., 2010) but also some successful attempts using "omic" approaches, namely transcription analysis by cDNA microarrays and PCR techniques (Manchado et al., 2008; Osuna-Jiménez et al., 2009; Prieto-Álamo et

al., 2009 and a few others) and proteomics (Forné et al., 2009; Salas-Leiton et al., 2009; Costa et al., 2010) as screening tools in mechanistic research, although most relate to aquaculture. Despite the growing focus on this species for environmental monitoring in SW Europe, comparable to the importance of flounder (*Platichthys flesus*) in northern Europe, few genomic resources are presently available posing serious constraints for surveys at genome-wide level. For such purpose the identification and characterization of genes responsive to environmental toxicants can be a valuable contribution for understanding underlying biological mechanisms and developing strategies with applications within ERA.

The main goal of the present work is to evaluate the transcription profiles of a set of toxicologically-relevant genes in a benthic fish of growing importance in the field of environmental toxicology as potential biomarkers of exposure to real sediment-based mixtures of contaminants. In addition, the regulation of the selected genes' transcription shall provide novel insights into the complex mechanistics of multi-class contaminants mixtures and will compare different types of fish bioassays for their assets and handicaps for ERA. For such purpose, the genes coding for five (out of six) response proteins were identified and partially characterized at the sequence level for the first time in this species and their transcripts analysed and integrated with sediment quality guidelines.

## 2. Methods and Materials

### *2.1. Study area and experimental design*

The study area (the Sado Estuary, W Portugal) consists of a large basin of great socio--economical importance. The estuary is historically subjected to many sources of anthropogenic pollution: urban (from the city of Setúbal); industrial (from the city's heavy-industry belt that includes chemical plants, a thermoelectrical unit, shipyards, ore deployment facilities and others) and from runoffs from the upstream agriculture grounds. A large part of the estuary is classified as a natural reserve and the area is also very important for tourism, fisheries and maritime transport. The conjugation of all these factors has dictated the need to enforce environmental monitoring in order to develop efficient environment management policies (refer to Caeiro et al., 2009, for an ecological risk assessment strategy for the estuary).

Three sites of the study area were chosen according to their geographical location (Fig. 4.3.1) and to previous research (e.g. Caeiro et al., 2009; Costa et al., 2009a). A reference (clean) site (R) was chosen for being the farthest from pollution sources. Two contaminated sites were selected, located at the city of Setúbal's important commercial harbour (site $C_1$) and off Setúbal's heavy-industry area (site $C_2$). Sediment collection and assays were performed on May 2007. Freshly-collected sediment samples were divided for the laboratory assays and chemical characterization (see below).

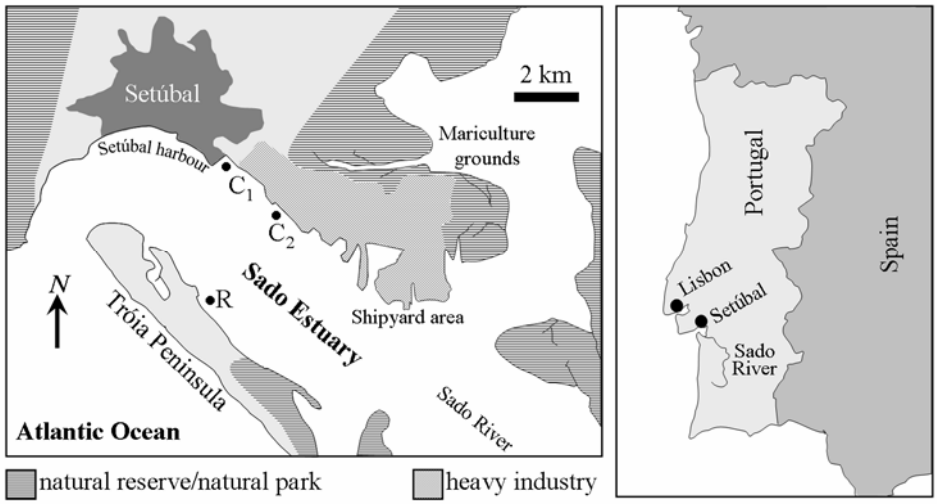


**Fig. 4.3.1.** Map of study area (the Sado Estuary, W Portugal) showing the relative position of the assay and sediment collection sites (•): R (reference) plus $C_1$ and $C_2$ (contaminated).

Juvenile hatchery-brood *Solea senegalensis* were exposed to sediments from the three sites through simultaneous laboratory and *in situ* 28-day bioassays (fish standard length = 61.0 ± 8.4 mm). The laboratory assays were performed in duplicate with a recirculation arrangement of 15-L capacity white polyvinyl tanks with blunt edges where 2 L of fresh sediments (providing a surface of approximately 525 $cm^2$) plus 12 L of clean seawater were allocated, following previous assays with the species (Costa et al., 2009a). The photoperiod was set at 12 h light:dark and a weekly 25 % water change was applied to ensure constancy of water quality parameters with minimal removal of suspended particles and dissolved contaminants: temperature was set at 18 ± 1 ºC, salinity = 32.1 ± 0.3, pH 8.0 ± 0.1, oxygen saturation = 56.5 ± 0.2% (supplied by constant aeration) and total ammonia was maintained within 1.6 ± 0.6 $mg.L^{-1}$ (the toxic unionized ammonia was restrained at 0.04 ± 0.02 $mg.L^{-1}$). Sediments were allowed to settle for 48 h before the beginning of the assay. Twenty randomly-selected animals were distributed per replica. The *in situ* assays were prepared using 90 × 90 × 30 cm PVC plastic cages lined with a 5 mm plastic mesh. Each cage was divided into two equal--sized compartments that were regarded as replicates. Twenty random-selection animals were allocated in each compartment. The cages were placed over the bottom at each site with scuba equipment in order to ensure direct contact with the sediment. Sampling was scheduled for days 0 ($T_0$), 14 ($T_{14}$) and 28 ($T_{28}$), with five individuals per replica being collected and euthanized by cervical sectioning prior to liver excision. Day 0 animals consisted of ten individuals retrieved from the rearing tanks.

*2.2. Sediment characterization and contamination profiles*

Sediments were characterized for redox potential (Eh), fine particle (< 67 μm) fraction (FF) and total organic matter (TOM) by electrode measurement (immediately after collection), hydraulic

sieving (after disaggregation with pyrophosphate and organic carbon combustion (at ≈ 500 ºC), respectively. Sediment inorganic (element) contaminants (arsenic, cadmium, chromium, copper, lead, manganese, nickel and zinc) were determined by inductively coupled plasma mass spectrometry after acid digestion in closed Teflon vials (Caetano et al., 2007). Total mercury was determined by atomic absorption spectrometry with pyrolysis and gold amalgamation according to Costley et al. (2000). The procedures were validated by analysis of the reference sediments MESS-2and PACS-2 (National Research Council, Canada) and MAG-1 (US Geological Survey, USA). Sediment PAHs was analysed by gas chromatography-mass spectrometry (GC-MS) after Soxhlet-extraction with an acetone+hexane (1:1 v/v) mixture as described by Martins et al. (2008). Organochlorines (PCBs and DDT plus its main metabolites) were determined by GC with electron capture detection (GC-ECD) after Soxhlet extraction with *n*-hexane and column fractioning, following (Ferreira et al., 2003). The quantification of organic contaminants was validated by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA).

*2.3. RNA isolation and cDNA synthesis*

Total RNA was isolated from ≈ 5-10 mg liver samples from three randomly-selected individuals from each test and sampling time with the spin column-based RNeasy Mini Kit (Qiagen), following instructions from manufacturer, and including a RNA clean-up step with the RNase-Free DNase Set (Qiagen) to eliminate residual genomic DNA. Total RNA was quantified and analysed for purity (assessed by the 260/280 nm absorbance ratio) with a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA quality was also verified by agarose gel electrophoresis. Aliquots containing the same amount of total RNA were pooled per experimental treatment and sampling time. cDNA was synthesized by reverse transcription from 1 µg pooled RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to manufacturer instructions.

*2.4. Primer design and cDNA sequencing*

Degenerate primers for *S. senegalensis* CYP1A (cytochrome P450 1A), MT1 (metallothionein 1), CAT (catalase), GPx (glutathione peroxidase 1) and CASP3 (caspase 3) cDNAs were designed based on the alignment of available teleost and *Rattus norvegicus* cDNA sequences deposited in GenBank public database using the software MEGA4 (Tamura et al., 2007). For sequencing, target gene amplification was done by standard PCR from pooled cDNA aliquots. Sequencing was done with a 3730 XL DNA analyzer (Applied Biosystems). CYP1A, MT1 and CAT were sequenced directly from PCR products but GPx and CASP3 required previous cloning in the M13 vector. After homology confirmation (Table 4.3.1) with database sequences using the software Blast (Altschul et al., 1990), the edited and trimmed cDNA sequences for CYP1A1, MT1, CAT, GPx and CASP3 genes were deposited in the GenBank (National Institutes of Health of the USA) public access data base (Table

4.3.2, see also Annex 2 for the full sequences). Specific primers were afterwards designed for real--time PCR. Primers for *S. senegalensis* HSP90AA (heat shock protein 90 kDa isoform alpha) and the housekeeping (reference) 18S (ribosomal subunit 18) genes were obtained from Manchado et al. (2008) and Cerdà et al. (2008), respectively. Gene selection was based on the potential function of respective proteins in response to toxicant-induced stress: anti-oxidative (GPx and CAT); metal--binding and detoxification (MT1); detoxification of organic contaminants (CYP1A); protein unfolding and chaperone (HSP90AA) and apoptosis (CASP3). Primer specificity, annealing temperature and amplicon size were confirmed by agarose gel electrophoresis (Table 4.3.2).

**Table 4.3.1.** Sequenced *S. senegalensis* target genes' complete cDNAs or ESTs and respective match scores.

| | **cDNA/mRNA sequence** | | **Similar to** | **Blast results** | | | |
|---|---|---|---|---|---|---|---|
| *Gene ID* | *Size (bp)* | *Status* | *Species* | *GenBank Accession* | *%ID* | *Score* | *e-Value* |
| CYP1A | 591 | Partial | *Paralichthys olivaceus* | EF451958.1 | 88 | 750 | 0.0 |
| MT1 | 183 | Complete | *Pleuronectes platessa* | X56743.1 | 90 | 254 | $8.0 \times 10^{-66}$ |
| GPx | 385 | Complete | *Oplegnathus fasciatus* | AY734530.1 | 86 | 490 | $1.0\times10^{-136}$ |
| CAT | 432 | Partial | *Oplegnathus fasciatus* | AY734528.1 | 90 | 523 | $2.0\times10^{-146}$ |
| CASP3 | 352 | Partial | *Danio rerio* | NM_131877.2 | 92 | 130 | $4.0\times10^{-11}$ |

**Table 4.3.2.** Primers used for quantitative real-time PCR and relevant data.

| **Gene ID** | **GenBank accession** | **Primer** | **Primer sequence (5' - 3')** | **Amplicon size (bp)** | **$T_{ann}$ (ºC)[§]** |
|---|---|---|---|---|---|
| CYP1A | GU946412 | Forward | TGGGCAGCAAACCTTACCTG | 213 | 60 |
| | | Reverse | CTGTGCTGAAGGCCAGACTC | | |
| MT1 | GU946410 | Forward | TTGCGAATGCTCCAAGACTG | 157 | 52 |
| | | Reverse | GTCGCATGTCTTCCCTTTAC | | |
| GPx | HM068301 | Forward | ATGAACGAGCTGCACTGTCG | 212 | 55 |
| | | Reverse | AGATAGACAAACAAGGGGTGTG | | |
| CAT | GU946411 | Forward | TGAGCAGGCTGAAAAGTTCC | 162 | 52 |
| | | Reverse | GGCATGTTACTTGGGTCAAAG | | |
| CASP3 | HQ115741 | Forward | CATCATCAACAACAAGAACTTTGACG | 181 | 59 |
| | | Reverse | ATGGTCTTCCTCCGAGGCTT | | |
| HSP90AA[†] | AB367526 | Forward | GACCAAGCCTATCTGGACCCGCAAC | 105 | 52 |
| | | Reverse | TTGACAGCCAGGTGGTCCTCCCAGT | | |
| 18S[‡] | AM882675 | Forward | GAATTGACGGAAGGGCACCACCAG | 148 | 55 |
| | | Reverse | ACTAAGAACGGCCATGCACCACCAC | | |

[†]From Manchado et al. (2008).

[‡]From Cerdà et al. (2008).

[§]Primer annealing temperature set for real-time PCR.

*2.4. Quantitative real-time PCR*

Quantitative real-time PCR was performed on a 96-well LightCycler 480 II Instrument using the SYBR Green I Master Mix for real-time PCR (all from Roche Applied Science). Each reaction (20 µL) was prepared with 10 µL Master Mix, 7 µL PCR grade ultra-pure water, 1 µL of each primer's 10 µM solution and 1 µL template cDNA sample. All analyses were performed in triplicate. The gene for the ribosomal protein 18S was considered for normalization. The PCR efficiencies (*E*) and relative transcription of the target genes were calculated as described by Pfaffl (2001). Accordingly, efficiency is estimated as:

$$E = 10^{-\frac{1}{slope}} \quad [1]$$

The *slope* is returned from the calibration curves for each target gene, obtained from six serial dilutions of a pooled cDNA sample (lowest $r^2$ = 0.98). According to Pfaffl (2001), the *E* values for each target gene were used to compute the relative transcription (*RT*) for exposed fish (*sample*) relatively to fish collected at $T_0$ (*calibrator*), which is described to the ratio:

$$RT = \frac{\left(E_{target}\right)^{\Delta C_t(calibrator-sample)}}{\left(E_{reference}\right)^{\Delta C_t(calibrator-sample)}} \quad [2]$$

Where $C_t$ represents the threshold cycle and $E_{reference}$ the efficiency for the reference gene 18S. $T_0$ animals were selected as the calibrator group for statistical purposes. The $C_t$ estimates and primer melting curves (for confirmation of specific amplification) were obtained using the LightCycler 480 II manufacturer software.

*2.5. Estimation of apoptotic indices*

Apoptotic cells in fish hepatic parenchyma were identified through the TUNEL reaction (TdT--mediated dUTP-X nick end labelling) using the *In Situ* Cell Death Detection Kit with fluorescein as fluorochrome (Roche Applied Science) from paraffin-embedded sections, as indicated by the manufacturer. In brief: fresh liver portions from three randomly-selected individuals from each test and sampling time (including $T_0$ animals) were fixed in Bouin-Hollande's fixative, dehydrated in a progressive series of ethanol and embedded in paraffin (xylene was used for intermediate

impregnation). Sections (3 μm thick) were dehydrated, permeabilized with a proteinase K (Invitrogen) solution (20 $\mu g.mL^{-1}$ for 30 min at 60 ºC) and stained with the TUNEL reaction for 1 h at 37 ºC. Analyses were performed with a DMLB model microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps and fitted with an I3 filter for fluorescein (Leica Microsystems). Observations were made from at least eight sequential sections on each slide. The apoptotic indice (AI) was estimated as the number of apoptotic (TUNEL-positive) cells per section $mm^2$. The identification of apoptotic cells was based on the intensity of fluorescence and morphological criteria (after Häcker, 2000).

*2.6. Statistics and integration of data*

The sediment contaminants' potential to cause adverse effects to organisms was determined by comparison of the measured concentrations with the Threshold Effects Level (*TEL*) and the Probable Effects Level Sediment Quality Guidelines (MacDonald et al., 1996). The overall potential impact of each class of contaminants was inferred from the Sediment Quality Guideline Quotient (*SQG-Q*), which was calculated as defined by Long and MacDonald (1998) through the formula:

$$SQG - Q = \frac{\sum_{i=1}^{n} \frac{C_i}{PEL_i}}{n} \qquad [3]$$

Where $C_i$ is the measured concentration of the $i^{th}$ contaminant, $PEL_i$ the available *PEL* guideline for the contaminant *i* and *n* the number of contaminants in the considered class or group. Each tested sediment was given an overall rating according to the *SQG-Q* combining all contaminant classes as proposed by MacDonald et al. (2004): *SQG-Q* < 0.1 - unimpacted; 0.1 < *SQG-Q* < 1 - moderately impacted; *SQG-Q* > 1 - highly impacted.

By failing to comply with the parametric ANOVA assumptions of homocedascity and normality of data, the non-parametric Mann-Whitney *U*-test was employed to assess significant differences in gene expression and apoptotic indices between fish exposed to the contaminated sediment $C_1$ and $C_2$ and fish exposed to the reference sediment (R). The non-parametric Kruskall--Wallis ANOVA by ranks *H* statistic was computed to verify overall differences between laboratory and field-exposed fish. The non-parametric Spearman's rank-order correlation *R* statistic was employed to find correlations between gene expression data and the sediments' potential to cause adverse effects, given by the *SQG-Q* values. A significance level of $\alpha = 0.05$ was considered for all analyses. Statistics were performed according to Zar (1998), using the software Statistica (Statsoft Inc.). Cluster analyses on gene expression data were performed with the software DAnTE (Polpitiya et al., 2008).

## 3. Results

### *3.1. Sediment characterization*

The reference sediment (R) was confirmed to be overall unimpacted by xenobiotics, being the least anoxic and also the sediment with the lesser proportion of TOM and FF (Table 4.3.3). Sediment from site $C_1$ was found to be the most impacted, being the most contaminated by inorganic substances (metals and metalloids). Sediment $C_2$ was found the most contaminated by PAHs and organochlorines. However, both sediments $C_1$ and $C_2$ could be globally considered as moderately impacted. Arsenic (As) copper (Cu), total mercury (Hg), nickel, lead (Pb) and zinc (Zn) were the main elements of concern in the contaminated sediments whereas 4- and 5-ring PAHs were the most important organic xenobiotics. Organochlorine concentrations were higher in sediment $C_2$, with especial relevance for DDTs (with *pp*'DDT presenting a concentration above the *TEL* threshold).

### *3.2. Gene transcription analyses*

Different patterns of gene transcription were observed, not only between laboratory and *in situ* assays but also between animals collected at $T_{14}$ and $T_{28}$ (Fig. 4.3.2). Only the transcription patterns for CAT failed to reveal significant differences between laboratory and field exposed fish at both $T_{14}$ and $T_{28}$. The relative expression patters for CASP3 were significantly different between the two types of assays at both sampling times (Kruskall-Wallis $H$, $p < 0.05$). The transcription of the remaining genes revealed significant differences only at $T_{28}$ (Kruskall-Wallis $H$, $p < 0.05$).

For laboratory-tested animals collected at $T_{14}$, all genes were downregulated in fish exposed to sediment $C_2$, relatively to the reference treatment (exposure to the clean sediment, R). For the same assays at the same sampling point, only the MT1, CAT and HSP90AA genes were observed to be upregulated, in $C_1$-exposed animals. However, an opposite trend was observed after 28 days of laboratory exposure to the three sediments, with $C_1$ and $C_2$ test significantly eliciting upregulation of all genes' transcription comparatively to the reference exposure. The highest levels of transcription for the laboratory assays (comparatively to the reference treatment) were observed to occur in fish exposed to sediment $C_2$ for 28 days, namely for CAT and CYP1A (approximately 37- and 18-fold, respectively). Cluster analysis confirmed the different patterns of transcription between fish collected at $T_{14}$ and $T_{28}$ (Fig. 4.3.3). In addition, the relative transcription ratios were grouped in two distinct clusters, however, MT1 and CASP3, as well as CYP1A and CAT, were allocated in the same clusters at both sampling times.

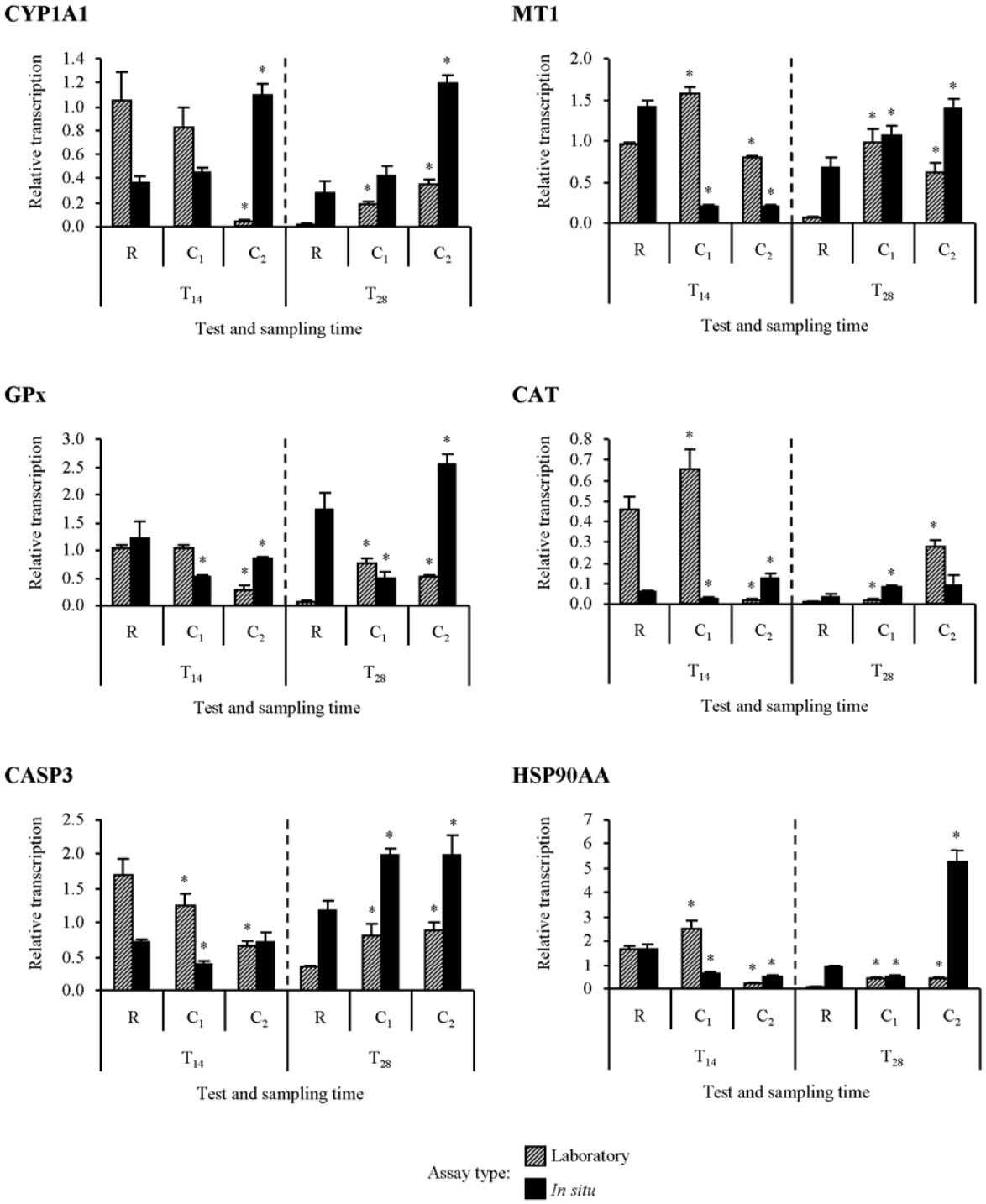


**Fig. 4.3.2.** Average relative gene transcription ± standard deviation (in arbitrary units) in the livers of *S. senegalensis* exposed to the reference (R) and contaminated ($C_1$ and $C_2$) sediments for CYP1A1, MT1, GPx, CAT, CASP3 and HSP90AA genes. [*] indicates significant differences from fish exposed to the reference sediment at the same assay type and sampling time ($n$ = 3; Mann-Whitney $U$, $p < 0.05$). Fish collected at $T_0$ are the calibrator group.

**Table 4.3.3.** General characterization and risk assessment (by comparison with available guidelines for the analysed contaminants) for the sediments collected from the three surveyed sites: R (reference) and $C_1$ plus $C_2$ (contaminated). The sediment quality guidelines *TEL* and *PEL* were obtained from Macdonald et al. (1996). Only the contaminant concentrations for which guidelines are available are shown.

| | | | ***Site*** | **R** | **$C_1$** | **$C_2$** | | |
|---|---|---|---|---|---|---|---|---|
| | | | TOM (%) | 2 | 10 | 7 | | |
| | | | FF (%) | 26 | 96 | 76 | | |
| | | | Eh (mV) | -140 | -300 | -312 | *SQGs* | |
| Contaminant | | | | | | | ***TEL*** | ***PEL*** |
| Inorganic (µg.g$^{-1}$ sediment dw) | Metalloid | As | | 5.20±0.10 | 23.98±0.48* | 20.69±0.41* | 7.24 | 41.6 |
| | Metal | Cd | | 0.06±0.00 | 0.26±0.01 | 0.29±0.01 | 0.68 | 4.21 |
| | | Cr | | 18.14±0.36 | 80.73±1.61* | 51.70±1.03 | 52.3 | 160 |
| | | Cu | | 28.20±0.56* | 172.72±3.45** | 95.31±1.91* | 18.7 | 108 |
| | | Hg† | | 0.11±0.00 | 0.69±0.01* | 0.71±0.01** | 0.13 | 0.7 |
| | | Ni | | 7.31±0.15 | 33.30±0.67* | 20.49±0.41* | 15.9 | 42.8 |
| | | Pb | | 18.57±0.37 | 55.19±1.10* | 43.76±0.88* | 30.2 | 112 |
| | | Zn | | 72.29±1.45 | 364.83±7.30** | 269.31±5.39* | 124 | 271 |
| | | | *SQG-$Q_{inorganic}$* | *0.16* | *0.79* | *0.58* | | |
| Organic (ng.g$^{-1}$ sediment dw) | PAH | 3-ring | acenaphthylene | 0.79±0.13 | 2.38±0.40 | 2.18±0.37 | 5.87 | 128 |
| | | | acenaphthene | 0.73±0.12 | 12.25±2.08* | 7.83±1.33* | 6.71 | 88.9 |
| | | | fluorene | 1.19±0.20 | 15.33±2.61 | 9.95±1.69 | 21.2 | 144 |
| | | | phenanthrene | 10.28±1.75 | 63.87±10.86 | 59.91±10.18 | 86.7 | 544 |
| | | | anthracene | 2.30±0.39 | 21.00±3.57 | 20.84±3.54 | 46.9 | 245 |
| | | 4-ring | fluoranthene | 23.34±3.97 | 315.71±53.67* | 345.24±58.69* | 113 | 1,494 |
| | | | pyrene | 21.51±3.66 | 263.18±44.74* | 286.33±48.68* | 153 | 1,398 |
| | | | benzo(a)anthracene | 3.70±0.63 | 81.25±13.81* | 93.99±15.98* | 74.8 | 693 |
| | | | chrysene | 2.35±0.40 | 41.06±6.98 | 46.68±7.94 | 108 | 846 |
| | | 5-ring | benzo(a)pyrene | 5.42±0.92 | 101.86±17.32* | 126.76±21.55* | 88.8 | 763 |
| | | | dibenzo(a.h)anthracene | 0.66±0.11 | 13.32±2.26* | 13.93±2.37* | 6.22 | 135 |
| | | | *SQG-$Q_{PAH}$* | *0.01* | *0.11* | *0.12* | | |
| | PCB | | Total PCBs | 0.80±0.14 | 7.91±1.34 | 11.97±2.04 | 21.6 | 189 |
| | | | *SQG-$Q_{PCB}$* | *0.00* | *0.04* | *0.06* | | |
| | DDT | | *pp*'DDD | < d.l. | 0.37±0.06 | 0.71±0.12 | 1.22 | 7.81 |
| | | | *pp*'DDE | < d.l. | < d.l. | 0.59±0.10 | 2.07 | 374 |
| | | | *pp*'DDT | < d.l. | < d.l. | 1.22±0.21* | 1.19 | 4.77 |
| | | | *SQG-$Q_{DDT}$* | *0.00* | *0.02* | *0.12* | | |
| | | | *SQG-$Q_{Organic}$* | *0.01* | *0.09* | *0.11* | | |
| | | | *SQG-$Q_{Total}$* | *0.06* | *0.33* | *0.28* | | |
| | | | Overall rating | **Unimpacted** | **Moderately impacted** | **Moderately impacted** | | |

[*] - values above *TEL*; [**] - values above *PEL*

†The provided mercury concentrations relate to total Hg (inorganic plus organic species).

*PEL* - probable effects level sediment quality guideline; *TEL* - threshold effects level sediment quality guideline; *SQG-Q* - sediment quality guideline quotients [3].

Regarding the *in situ* assays, exposure to the two contaminated sediments caused downregulation of MT1, GPx and HSP90AA at $T_{14}$ and of CAT and CASP3 only for test $C_1$.As for the laboratory assays, a different pattern was observed for animals sampled at $T_{28}$, with the exception of the CYP1A gene, which revealed a similar pattern between the two sampling times, with exposure to

sediment $C_2$ (the most contaminated by organic contaminants) eliciting a very significant transcriptional upregulation relatively to the reference, R, test (3-4 fold). The highest levels of transcription comparatively to R-tested fish during the *in situ* assays were observed for the HSP90AA gene after 28 days of exposure (≈ 6 fold). After 14 days of exposure, the relative transcription ratios could be grouped in two distinct clusters, the first including HSP90AA, MT1 and GPx genes and the second all the remaining genes (Fig. 4.3.3). However, as before, the correlations between relative gene transcription ratios revealed a different pattern at $T_{28}$, with CASP3 and GPx forming a separate cluster and the expression of HSP90AA appearing uncorrelated with the remaining.

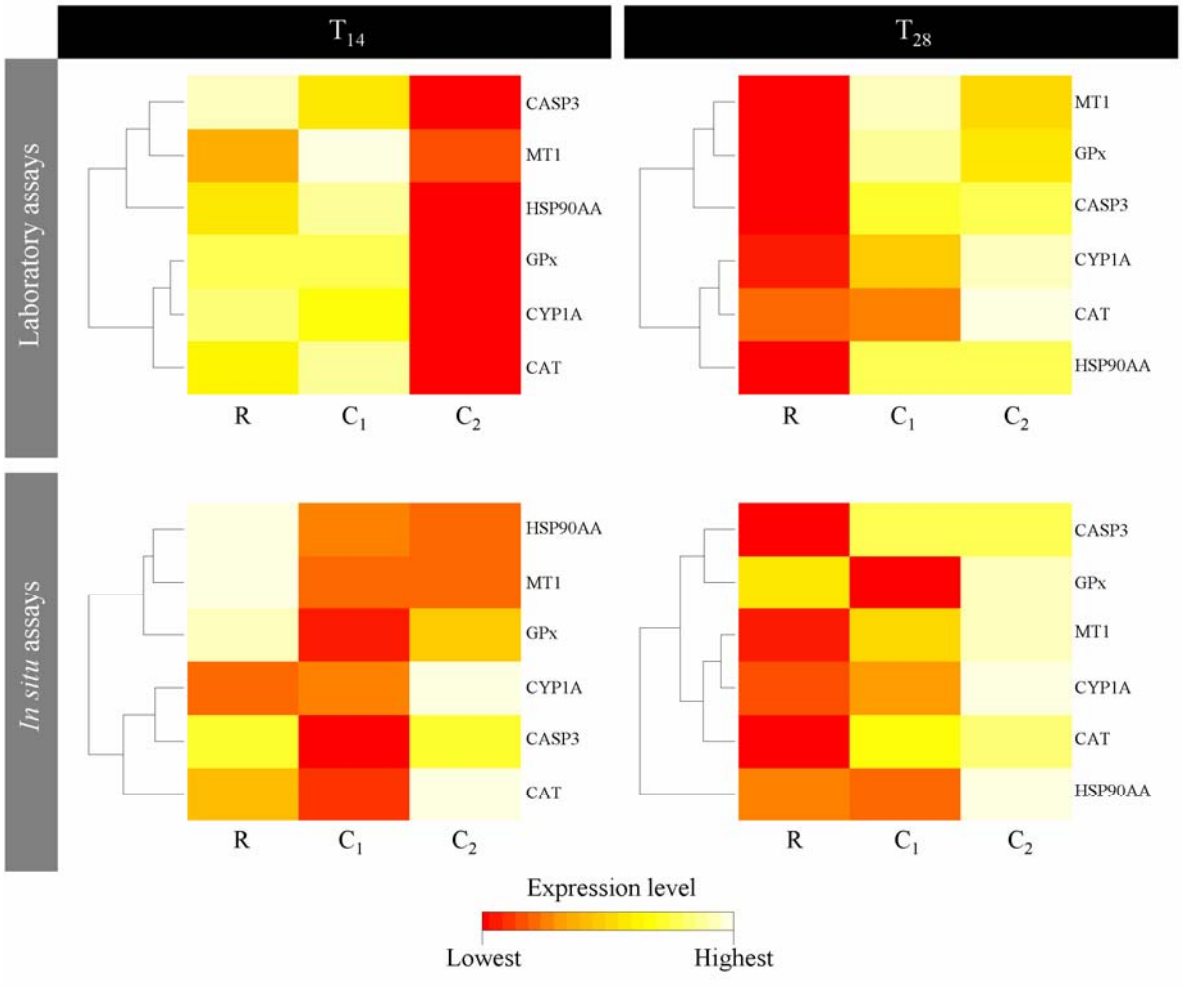


**Fig. 4.3.3.** Cluster analyses on the relative transcription of all surveyed genes for laboratory- and *in situ*- tested fish collected at $T_{14}$ and $T_{28}$. Complete linkage was employed as amalgamation rule and Euclidean distances as metrics.

### *3.3. Hepatocyte apoptosis*

A different pattern of hepatocyte apoptosis (Fig. 4.3.4A) was observed between laboratory and *in situ* tested fish. With the exception of a decrease in the number of apoptotic hepatocytes in fish exposed to sediment $C_1$ for 14 days, the field assays did not reveal any significant change in the

apoptotic indice of animals exposed to the contaminated sediments relatively to the reference test at either time point (Fig. 4.3.4B). However, laboratory exposure to the sediment $C_2$ increased liver cell apoptosis in animals exposed for 14 and 28 days, whereas apoptosis was found to be reduced in comparison to R-tested fish after 28 days of exposure to sediment $C_1$.

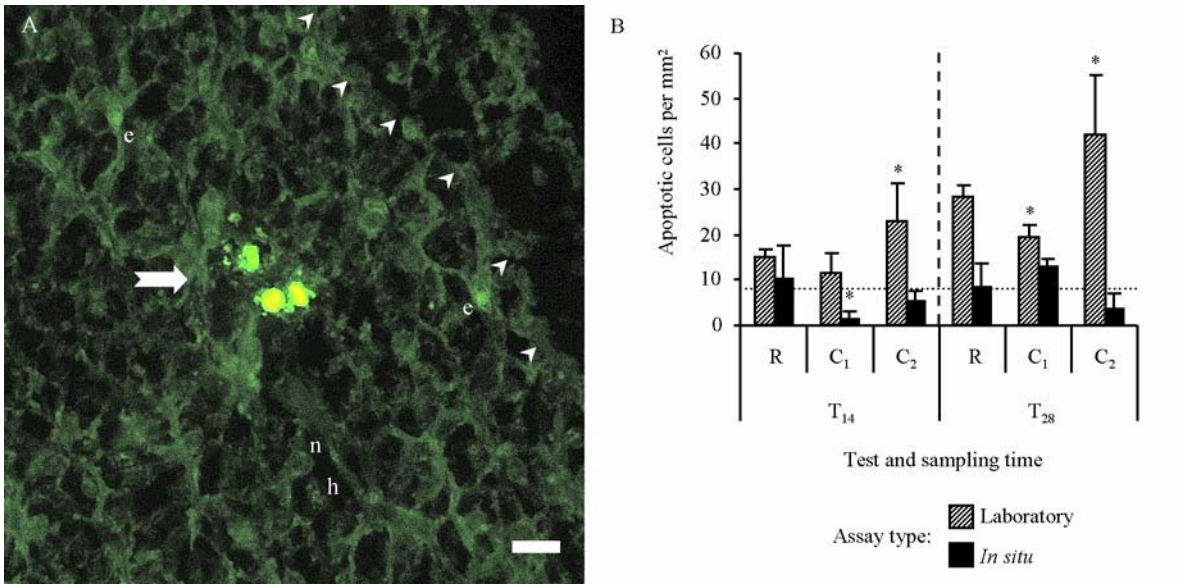


**Fig. 4.3.4.** Determination of hepatic apoptosis. A) Apoptotic (TUNEL-positive) cells in the hepatic parenchyma of a fish exposed in the laboratory to contaminated sediment $C_2$ (arrow), compared to normal hepatocytes (h) and a necrotic area (arrowheads). Erythrocytes inside sinusoid vessels can also be observed (e). B) Apoptotic indice (given by the number of apoptotic cells per section $mm^2$) variation during laboratory and *in situ* exposure to sediments R (reference), $C_1$ and $C_2$ (contaminated). The dotted line stands for the levels of apoptosis in $T_0$ fish and is provided for comparison purposes. [*] indicate significant differences from fish exposed to the reference sediment at the same assay type and sampling time ($n$ = 3; Mann-Whitney $U$, $p < 0.05$).

### *3.4. Correlating gene transcription to sediment risk*

Correlation analysis between the potential to cause adverse effects of each surveyed class of sediment contaminants (organic, inorganic and total) and the gene transcription plus AI data retrieved from the exposed fish revealed after 14 days of exposure that only CYP1A in field-tested fish was positively correlated to organic xenobiotics (Table 4.3.4). Also, the apoptotic indice estimated for the same assay and sampling time was positively correlated to the relative transcription of MT1, GPx and CASP3. Conversely, at $T_{28}$, CYP1A regulation was found to be correlated to the organic contaminants' *SQG-Q* for both types of assays, as well as CAT in laboratory-tested animals. Interestingly, the MT1 gene transcription was observed to be very significantly correlated to contamination by metals in laboratory-exposed fish (as well as GPx) and to organic contamination in field-tested animals, with the opposite trend being observed for CASP3. Also after 28 days of exposure, no significant correlations were found involving the AI in laboratory-tested animals but a significant negative correlation was found between the AI and GPx transcription in field-tested fish.

Again after 28 days of exposure, in the laboratory assays, transcription of HSP90AA was found to be positively correlated to both classes of contaminants' *SQG-Q*s and also to the global *SQG-Q*, whereas no significant correlation were for observed for *in situ*-tested fish.

**Table 4.3.4.** Spearman rank-order correlation *R* statistics between the relative transcription ratio of the surveyed genes, the hepatic apoptotic indice (AI) and the sediment quality guideline quotients (*SQG-Q*s) for the surveyed classes of sediment contaminants.

| Sampling time | | Laboratory assays | | | | *In situ* assays | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AI | $SQG\text{-}Q_{Total}$ | $SQG\text{-}Q_{Inorganic}$ | $SQG\text{-}Q_{Organic}$ | AI | $SQG\text{-}Q_{Total}$ | $SQG\text{-}Q_{Inorganic}$ | $SQG\text{-}Q_{Organic}$ |
| $T_{14}$ | | | | | | | | | |
| | CYP1A | - | - | - | -0.84 | - | - | - | 0.90 |
| | MT1 | -0.95 | - | - | - | 0.69 | -0.69 | -0.69 | -0.74 |
| | CAT | -0.95 | - | - | - | - | - | - | - |
| | GPx | -0.71 | - | - | -0.71 | 0.95 | -0.95 | -0.95 | - |
| | CASP3 | - | - | - | -0.95 | 0.69 | -0.69 | -0.69 | - |
| | HSP90AA | -0.95 | - | - | - | - | - | - | -0.95 |
| | AI | | - | - | - | | -0.84 | -0.84 | - |
| $T_{28}$ | | | | | | | | | |
| | CYP1A | - | - | - | 0.95 | - | - | - | 0.90 |
| | MT1 | - | 0.95 | 0.95 | - | - | - | - | 0.95 |
| | CAT | - | - | - | 0.95 | - | - | - | - |
| | GPx | - | 0.95 | 0.95 | - | -0.95 | - | - | - |
| | CASP3 | - | - | - | 0.81 | - | 0.79 | 0.79 | - |
| | HSP90AA | - | 0.69 | 0.69 | 0.74 | -0.95 | - | - | - |
| | AI | | - | - | - | | - | - | - |

## 4. Discussion

The results revealed that the transcription of toxicity-related genes' transcription can be highly dependent on the time of exposure and assay type. However, even when surveying changes to gene transcription in soles exposed to mixtures of contaminants, as in the present study, the relative regulation of the surveyed genes could be positively correlated to the levels of generalist classes of sediment-bound contaminants although at a later stage of exposure. This time-of-exposure factor is likely related to the different phases of xenobiotic-induced stress, an issue that has long been regarded of great relevance in ecotoxicology (see for instance Triebskorn et al., 1997). On the other hand, the observed differences between the gene transcription profiles of laboratory and *in situ* bioassays probably derived from two main factors: i) differences in bioavailability and ii) confounding factors that affected the experimental procedure, especially in the field bioassays.

Past research already reported differences between laboratory and *in situ* assays and the resulting difficulties to determine cause-effect relationships (for instance Hatch and Burton, 1999). Still, the causes of such differences are not easy to pin-point. The bioavailability of inorganic and organic xenobiotics is known to be favoured by disturbance events, combined with high TOM and FF that act as a trap for contaminants and changes in sediment pH and redox status when they are re--exposed to an oxygenated medium (Eggleton and Thomas, 2004; Atkinson et al., 2007). It is therefore highly probable that disturbance caused by sediment collection, handling and the animals' own scavenging activity in the test tanks, combined with constantly aerated water, favoured the release of contaminants to the water column, turning them more readily available to organisms. No noticeable sediment disturbance events were observed at the two most contaminated sites ($C_1$ and $C_2$) but field exposure at the reference site probably introduced some degree of experimental noise related to access to food items. In fact, whereas small molluscs were often found in the digestive tracts of animals caged in sites $C_1$ and $C_2$ (of low hydrodynamics), animals allocated at the reference area and collected at $T_{28}$ contained few food items, which is likely due to the observed partial sediment washing-off from beneath the cages, making it difficult for the fish to scavenge for preys. Pascual et al. (2003) found that food deprivation in fish increase hepatic oxidative stress and increase the activity of GPx (but not catalase), precisely the gene whose upregulation was stronger, in field reference-tested fish.

### *4.1. Different stages of stress are reflected in distinct patterns of gene transcription*

The relative gene transcription analysis showed that time of exposure in a bioassay-based survey is a decisive factor. It is long known that, when exposed to a continuous pressure by stress factors, organisms develop their biological responses through a series of steps, or phases, starting from disturbance, to restitution and response and ending in an adjustment phase that should either lead to adaptation or ultimately to chronic disease. This stress phase model applies also to gene expression responses, each phase presenting a unique up- or downregulation pattern of the same genes (Steinberg

et al., 2008). Fish exposed to either contaminated sediment ($C_1$ or $C_2$) revealed very distinct patterns on the surveyed genes' transcription after 14 and 28 days of exposure, often resulting in downregulation at $T_{14}$ followed by very significant upregulation at $T_{28}$, which can be even further enhanced when converting the relative transcription ratios (*RT*s) to fold variation relatively to animals exposed to the reference sediment (Fig. 4.3.5). These findings indeed suggest that sampling after 14 and 28 days of exposure reflect two distinct phases of stress, likely induced by sediment contamination (at least in most part), more notorious in laboratory-tested fish. It thus seems likely that during the first half of the assays the animals' ability to respond to environmental stress was impaired, which is reflected in the relative gene transcription of animals exposed to the contaminated sediments. From the assays' mid-term onwards, fish were most likely able to achieve some degree of metabolic balance, recover their hepatic metabolic machinery and increase the *RT*s (and probably expression) of important detoxification or xenobiotic-response related genes.

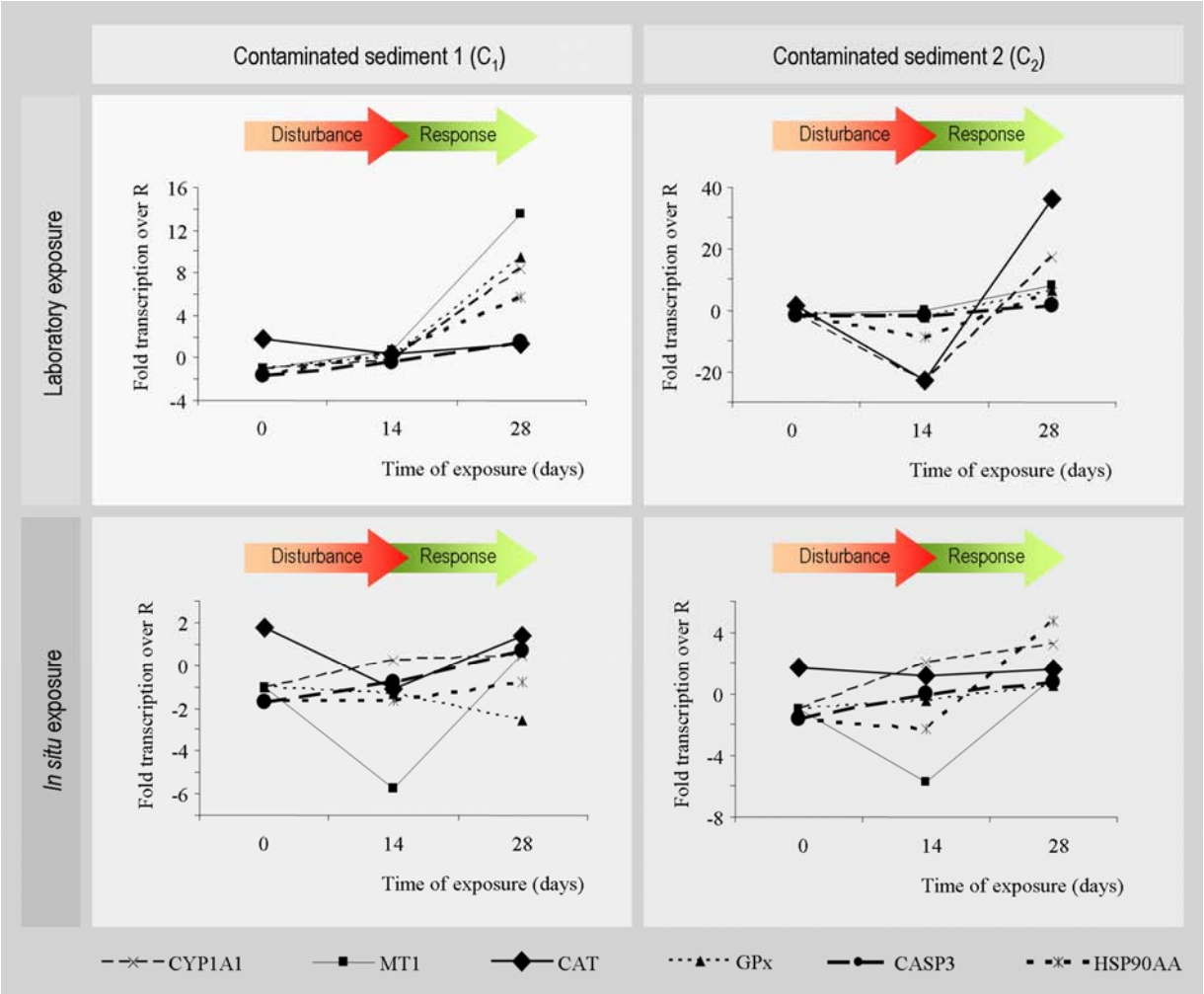


**Fig. 4.3.5.** Fold change in hepatic gene transcription relatively to fish exposed to the reference sediment (R) for laboratory and *in situ* tested *S. senegalensis*, exposed to sediment $C_1$ (the most contaminated by metals) and sediment $C_2$ (the most contaminated by PAHs and organochlorines), and its relation to the disturbance and recovery/response phases of stress.

Regarding laboratory assays, the first phase (0-14 days of exposure) resulted in either absence of a clear upregulation of the surveyed genes (in animals exposed to sediment $C_1$) relatively to the reference-tested organisms or a marked downregulation, as for the CYP1A and CAT genes in animals exposed to sediment $C_2$. Interestingly, these are two oxidative stress-related genes related to phases I and II of detoxification, respectively, that were observed to be very significantly upregulated in the livers of $C_2$-exposed fish for 28 days in the laboratory. In addition, the relative transcription of both genes was found to be consistently correlated in the laboratory assays. These findings are in accordance with the positive correlations found between the *SQG-Q*s for organic contamination for CYP1A (in both field and laboratory) after 28 days of exposure, since these contaminants, especially PAHs (best represented in sediment $C_2$) are detoxified by the CYP1A monooxygenases complex, which converts these liposoluble compounds into more hydrophilic ones (more easily excreted) with the production of ROS (e.g. Lemaire and Livingstone, 1997). In the field assays, the cleavage between the "disturbance" and "recovery/response" phases is more evident for MT1 and CAT genes in test $C_1$ and for HSP90AA and, especially, MT1 in the tests with sediment $C_2$.

*4.2. Transcription of CAT and GPx oxidative stress-related genes is linked to different classes of contaminants*

The transcription of both GPx and CAT genes has already been found to be enhanced in the teleost liver by exposure to either metallic (e.g. Hansen et al., 2007) or organic contaminants (e.g. Nahrgang et al., 2009). In the present study, however, the relative number of transcripts of the oxidative stress-related genes GPx and CAT, after 28 days of exposure, were observed to be correlated with different classes of contaminants in the laboratory assays, while in the field assays no significant correlations with the *SQG-Q*s could be retrieved for both. Transcription of GPx was found to be correlated with metallic contaminants while CAT was linked to organic contaminants (which may reveal a link with CYP1A transcription). Since catalase is a chief peroxisomal enzyme, involved in the reduction of hydrogen peroxide, it is possible that CAT induction in the laboratory-exposed animals reflects the direct or indirect formation of this ROS during PAH catabolism, which is in accordance, for instance, with the findings of Bilbao et al. (2010) in mullets injected with the carcinogenic 5-ring PAH benzo[a]pyrene. It should be noted, though, that some authors argued that CAT activity might not be a suitable biomarker in ERA for providing very variable and difficult to interpret responses, with some evidence that many, if not most, laboratory studies failed to provide convincing results (van der Oost et al., 2003). Few studies focused on CAT transcription as a potential biomarker, nevertheless, Nahrgang et al. (2009), found CAT mRNA level to be a much more consistent biomarker than the enzyme's activity in cods injected with B[a]P. The results suggest that CAT transcription has some potential as an indicator of exposure to sediment-bound organic contaminants even when metal+organic xenobiotic mixtures are involved, at least under experimental conditions that may enhance bioavailability. Still, the lack of any significant correlation with the *SQG-Q*s for the field

assays (although significant upregulation was observed in $C_1$ and $C_2$-tested fish) suggest some caution is needed to interpret these results when multiple confounding factors are at stake. The transcription of GPx, on the other hand, was found to be correlated to the MT transcription in laboratory-exposed fish for 28 days (refer to Fig. 4.3.3), and both linked to sediment contamination by metals (Table 4.3.4).

*4.3. Increased transcription of CYP1A gene as an indicator of exposure to organic contaminants*

Although one of the most consistent responses, especially in the field assays, with respect to global organic contamination, CYP1A transcription is clearly not entirely dependent on the presence of known inducers, such as PAHs, since exposure to sediment $C_1$, almost as contaminated by these substances as $C_2$, failed to promote a marked transcription upregulation. Besides the differences in the sediments geochemistry and their influence on contaminant remobilization (which differs for organic and inorganic contaminants), it is likely that the combination of metallic and organic xenobiotics had a considerable effect. In fact, in the past few years, a considerable literature sprung reporting that inhibitory or antagonistic effects between contaminants or classes of contaminants modulate the expression of CYP1A. The induction of CYP1A mRNA in the teleost liver has already been reported to be inhibited by metals and metalloids (like Cr, Ni, and As, well represented in sediments, especially $C_1$) in the presence of strong inducers like the aryl hydrocarbon receptor (Ahr) agonist B[a]P (being the Ahr-B[a]P one of the best known transcription factors for the CYP1A gene), without, however, affecting the basal level of expression (Sorrentino et al., 2005). Similar results have already been found, for example, in human cells exposed to B[a]P and arsenate (Spink, 2002). This information is in accordance with the present results considering that sediment $C_1$ is more contaminated by metals and arsenic than $C_2$ (although both have proximate levels of PAHs) and that exposure to sediment $C_2$ elicited the most significant upregulation of this gene's transcription. Other authors also reported an inhibitory effect on CYP1A induction in fish liver by organic compounds, including some PAHs like fluoranthene (Willet et al., 2001) and even after prolonged exposure to PCBs (Celander et al., 1996). Inhibition of CYP1A overexpression by organic xenobiotics is unlikely to have occurred in $C_1$-tested fish due to proximal levels of PAH between the two most contaminated sediments (as for fluoranthene, for instance) and the reduced concentrations of organochlorines in $C_1$ compared to sediment $C_2$. Nonetheless, flatfish have been reported to rapidly catabolize PAHs (Varanasi and Gmur, 1981), which is consistent with the overall strong correlations found between the transcription of the *S. senegalensis* CYP1A gene and the potential risk of the surveyed levels of sediment PAHs, confirming that, in spite the presence of multiple-class contaminants, measuring CYP1A transcripts retains an important value as a biomarker of exposure to CYP-inducing substances as PAHs, potentially producing results that can be better related to sediment CYP inducers than measuring CYP protein content (see for instance Costa et al., 2009a).

*4.4. The role of MT1 and HSP90AA as general- and oxidative stress proteins*

Metallothioneins (MTs) are small (≈ 6-7 kDa) cysteine-rich cytosolic proteins long known to be involved in metal and metalloid detoxification by sequestering metallic ions. For such reason, MT induction has been surveyed and employed as a potential biomarker of metal exposure (see Romero--Isart and Vašak, 2002, for a review). However, much research discloses either a failure to effectively correlate MT induction with environmental contamination by metals (e.g. Mouneyrac et al., 2002) or in fact reports its upregulation by organic contaminants. In a previous work from our group it was observed that MT induction in the livers of Senegalese soles was correlated to sediment PAHs (Costa et al., 2009a). Still, the majority of research performed on MT induction in aquatic organisms for the purpose of ecological risk assessment focus on MT-like protein content and not its gene transcription. The present results indicate that MT mRNA levels can very significantly correlate to either sediment metal+total contamination or organic contamination after 28 days of exposure, depending on assay type. This difference between laboratory and field assays is most likely caused by two main factors: i) the differential bioavailability of metals and ii) increased induction of MT by oxidative stress originated by organic contaminant detoxification processes. In fact, MT mRNA upregulation has been found to occur as a consequence of very different types of stressors in fish liver, even including thermal stress (van Cleef-Toedt et al., 2001). All this information appears to confirm that the induction of MT transcription might be, rather than a suitable biomarker for metal exposure, a good general indicator of stress, a role that has already been proposed by other authors (e.g. Viarengo et al., 1999).

Besides their task in the balance of intracellular metallic ions (including storage and detoxification), metallothioneins have also been reported as important scavengers of oxidative radicals. Tamai and co-workers (1993) have provided evidence that MTs can even functionally replace the important anti-oxidative copper-zinc enzyme superoxide dismutase (Cu-Zn SOD) and inclusive have their transcription greatly enhanced in SOD-deficient cells. On the other hand, it has been reported that MT is induced in the mouse model by $H_2O_2$, in a dose dependent response, as well as zinc, which is caused by the fact that MT transcription is not solely depending on the metal-responsive transcription factor (MTF) that binds to the metal response element (MRE) of the gene but also on other upstream transcription factors that are sensitive to oxidative radicals that are involved in complex, not yet fully understood, signalling pathways (Andrews, 2000; Laity and Andrews, 2007). Oxidative radicals produced as a consequence of CYP activity on organic toxicants should have triggered MT1 upregulation, thus explaining the higher transcription in the livers of fish exposed to sediment $C_2$ (most contaminated by organic contaminants), in the field, for 28 days, when compared to animals exposed to sediment $C_1$ (most metal-contaminated). In field-fish exposed for 28 days, MT1, CYP1A and the anti-oxidant phase II enzyme CAT (dedicated to $H_2O_2$ detoxification) were found to be correlated (Fig. 4.3.3), which is in accordance with this hypothesis. In the laboratory assays, however (where MT1 expression could be correlated to the metal *SQG-Q*), $MT_1$ formed a very clear cluster with GPx, excluding CYP1A or CAT, which indicates that MT induction was mostly caused by

metals. This correlation can be easily verified since the *SQG-Q*s for metals can be represented by the following ascending sequence: $R < C_2 < C_1$, precisely the same as the MT relative expression obtained for this test.

It is likely that the increased metal bioavailability afforded by the laboratory assays also contributed, at some extent, to increased MT mRNA levels. Some authors have inclusively argued that MT induction may lose its value in absence of high concentrations of good inducers (e.g. Jessen-Eller and Crivello, 1998; Mouneyrac et al., 2002). On the other hand, other authors reported that MT gene transcription in fish liver may only occur after prolonged exposure to low or moderate levels of waterborne metals even when strong inducers (such as Cd) are involved (Gonzalez et al., 2006). It should be noted though, that previous research from our group performed with *S. senegalensis* laboratory exposed to sediment collected at proximate locations failed to correlate MT protein contents with overall sediment contamination profiles (Costa et al., 2009a). Overall, the present findings suggest that MT gene transcription is likely to have a better potential as a biomarker of exposure than the translated product. It is possible, in fact, that protein contents do not entirely correlate with mRNA levels, e.g. by action of cysteine proteinases produced in affected organs (Costa et al., 2010), which is yet another indicator that gene expression, from translation to the final protein product, is affected by many factors. In addition, it is clear that MT translation or even full expression requires a careful interpretation since it may loose its specificity to metals at low-moderate metal exposure. Still, MT1 gene transcription can be a valuable indicator of general stress imposed by mixtures of contaminants due to its link to oxidative stress.

Heat shock proteins (HSPs) are cytosolic proteins that owe their name to the fact that they were first described in *Drosophila* after being induced by increased temperatures. Nowadays, they are recognized as multi-function chaperone proteins involved in protein folding. Surveying HSP expression in fish as a potential biomarker for stress (HSP90 and, especially, HSP70 are the most considered HSPs) has long been proposed for environmental toxicology (Basu et al., 2002; van der Oost et al., 2003). Nevertheless, only a relatively small number of published articles focused on HSP induction as a potential biomarker of exposure to chemical stressors in fish, occasionally reporting inconclusive results. Eder et al. (2009), for instance, found HSP90 gene expression to be significantly elevated in the livers of salmons exposed to pesticides whereas Letcher et al. (2010) describe no alterations in HSP content or transcription in the livers of charrs exposed to organochlorines, although HSP70 and 90 contents could be elevated in the brain. Osborne et al. (2007) have inclusively found a correlation between HSP90 induction and exposure to the endocrine disruptor 17α-ethynylestradiol in trout hepatocytes.

In general, there is a great deal of information missing regarding the mechanisms of HSP action and induction in the cells and their validation as potential biomarkers of exposure. Still, the present results indicate that there is some potential of these proteins and respective mRNA quantities as an indicator of general xenobiotic-induced stress since the relative transcription of the HSP90AA (termed inducible HSP90 - see Manchado et al., 2008) gene was found to be positively correlated to

all surveyed *SQG-Q*s (respective to total, metallic and organic sediment contamination) in laboratory--exposed fish for 28 days, i.e., after the disturbance phase of stress (Table 4.3.4). It is possible though, that HSPs transcription, and HSP90 in particular, respond more clearly to prolonged exposure and to higher concentrations of bioavailable contaminants, as it is likely to have occurred in the laboratory--assays.

It should also be noticed that, among their multiple (and not fully understood) functions, mammalian HSP90 proteins are also known to take part in CYP1A transcription by being one of the chaperones (as dimers) of the aryl hydrocarbon receptor (Ahr) - HSP90- immunophilin-like X--associated protein 2 (XAP-2) heterodimer, being important at least to enforce the adequate conformation of the ligand-binding site of the Ahr (Petrulis and Perdew, 2002). More recently, the importance of HSP90 for Ahr-mediated transcription of the CYP1A gene has been discovered in teleost hepatocytes (Wiseman and Vijayan, 2007). Although only in laboratory-tested fish for 28 days a correlation was found between CYP1A and HSP90AA was observed (Fig. 4.3.3), the relative expression of both genes was higher at $T_{28}$ for fish exposed to sediment $C_2$ (the most contaminated by organic substances), regardless of assay type. Although the exact significance of HSP90 upregulation remains unclear in the Ahr-mediated CYP1A transcription pathway it is likely that the increased transcription of the HSP90AA gene was modulated, in part, to respond to the need to detoxify organic xenobiotics. In fact, it is probable that the transcription of this gene can be modulated by oxidative stress induced by inorganic or organic substances, hence its potential value as a general indicator of xenobiotic-induced stress.

### *4.5. Apoptosis and the transcription of caspase 3*

Caspase 3 (cysteine-aspartic acid peptidase 3) is a key peptidase in caspase-dependent apoptosis, either through the intrinsic of external pathway. However, it must be noted that the induction of the caspase 3 protein does not act as an apoptosis trigger by itself, since this protein, as procaspase 3, needs activation (by cleavage at specific target sites) by the initiator caspases 8 (external pathway) or 9 (intrinsic, mitochondria-dependent pathway) to become the effector caspase 3 (refer to Porter, 2006, and Wyllie, 2010, among the many reviews on the topic). Apoptosis (a programmed cell death pathway, PCD, in animals) is a response mechanism that can be regarded as a controlled dismantlement of damaged cells to avoid the dissemination of mutations, hazardous cellular debris and the inflammatory response that typically follows necrosis and that may trigger neoplasic disease itself (Mantovani et al., 2008). Thus impairment of the apoptotic response is known to disrupt the balance between liver cell death and proliferation, promoting neoplasic development (Hoffmann et al., 2007). It has long been suspected that oxidative stress, in particular CYP1A-genererated ROS as a consequence of PAH catabolism (activation) is an important upstream trigger of apoptosis (for instance Holme et al., 2007), as well as oxidative radicals produced on the onset of metal-imposed toxicity (as Risso-de Faverney et al., 2001, reported for Cd). Nevertheless, there are several pathways

of intercellular and intracellular signalling that trigger apoptosis and, furthermore, it has been recognized that certain contaminant interactions can actually impair the process, with prejudice to tissue recovery and avoidance of neoplasic disease. For instance, the impairment of apoptosis by the interaction between metals and PAHs has already been described (e.g. Costa et al., 2010).

Hepatocyte apoptosis in flatfish has been reported to be induced by various inorganic and inorganic xenobiotics, however, there is evidence that this defence mechanism is replaced at higher concentrations of the toxicants by necrosis (Piechotta et al., 1999). The present study showed that *in situ* bioassays elicited few changes in hepatocyte apoptosis relatively to the reference test and, inclusively, to the basal levels of apoptosis ($T_0$ fish) at any sampling time-points (except for exposure to sediment $C_1$ for 14 days, with a significant decrease in the apoptotic indice). This information is accordance with the premise that laboratory bioassays greatly enhanced bioavailability and raised the basal level stress induced to the fish, therefore inducing apoptosis as the livers strived to recover from damage. Still, a significant decrease in the apoptotic indice was observed in fish exposed to sediment $C_1$ for 28 days that can be explained either by metallic-organic xenobiotic interactions or by general failure to cope with enhanced toxicity. In fact, many necrotic foci of variable extension were found in the hepatic parenchyma of these animals.

Most significantly, transcription of the CASP3 gene was found to be little related to the induction of apoptosis, except in field-assayed fish after 14 days of exposure. Still, CASP3 transcription could be correlated to *SQG-Q*s after 28 days of exposure for both types of assays, although to different classes of contaminants, similarly to what was observed for MT1 transcription. Metallothionein induction has actually been found to inhibit apoptosis, presumably by scavenging oxidative radicals (Kondo et al., 1997). It must be at this point recollected that transcription of MT1 and CASP3 were consistently linked in laboratory-exposed fish (Fig. 4.3.3), which could mean that the regulation of both genes is responding to the same stimulus, namely oxidative stress. Since exposure to sediment $C_1$ (most metal-contaminated) in the laboratory caused a very significant increase in MT1 relative expression, is it probable that this protein contributed to the decreased levels of hepatocyte PCD observed in this test, in spite of CASP3 upregulation, to which likely contributed the more moderate increase in the relative number of transcripts observed for the GPx and CAT anti-oxidative stress enzymes.

Apoptosis is, however, influenced by many other enzymes besides those directly related to oxidative stress. Amongst their varied functions, heat-shock proteins, for instance, are known to counter balance apoptosis, including HSP90, which inhibits apoptosis by interfering with the protein kinase B (Akt) pathway (see Takayama et al., 2003, for a review). This function for HSP90 is supported by the negative correlation found between this gene's relative expression and the apoptotic indices in fish exposed in the laboratory for 14 days and in the field for 28 days (see Table 4.3.4). In fact, fish field-exposed for 28 days to sediment $C_2$ revealed both the highest relative transcription of the CASP3 and HSP90AA genes, without any significant change in hepatocyte apoptosis comparatively to the reference treatment (Fig. 4.3.4B).

*4.6. Concluding remarks*

Gene transcription analysis is a more baseline approach to assess exposure biomarker than traditional methods based on enzyme content and activity since transcription is located upstream in the gene expression process, which starts with xenobiotic stimuli and ends with the protein final conformation and activity. This way, mRNA levels are not affected by proteolysis, denaturation or even functional group inactivation, for instance by competition of non-essential ions (such as toxic metals) for binding sites, as post-translation products are prone to be (even though transcript levels can be acted upon by pot-translation mRNA degradation mechanisms such as those based on small interfering RNAs). Not only the present results confirm the adequacy of transcriptomics in a species that is gaining focus in ecological risk assessment but also the effectiveness of assessing the surveyed genes' changes in transcript regulation in predictive studies where multiple sediment contaminants are involved, an issue that often results in responses difficult to interpret and to relate with environmental pollutants when more classical approaches are enforced. It is the particular case of CYP1A and even MT1, if this latest is to be regarded as a general stress indicator. The transcription of the GPx and CAT genes requires caution in its interpretation since oxidative stress has multiple causes (including from confounding factors). However, its survey can be correlated to contamination and to the transcription of other genes, thus constituting an important tool for predictive and mechanistic environmental toxicology. Although hepatic apoptosis could difficultly correlate to sediment risk, the regulation of a key gene in one of the most important PCD pathways (caspase 3) revealed to have a good potential as an unspecific biomarker of exposure, at least in benthic fish, since it successfully distinguished the two contaminated sediments from the reference for both types of assays. It would be of great interest to survey CASP3 regulation in aquatic organism exposed to further concentrations of sediment contaminants and different xenobiotic mixtures. Similarly, HSP90 has shown some potential as an indicator of xenobiotic-stress, regardless of its unspecificity.

It is crucial to consider the time-of-exposure factor when collecting data from bioassays, due to the clear existence of distinct phases of stress that are reflected in very different gene expression patterns. The results indicate that a shorter-term exposure may retrieve results inconsistent with the degree of contamination if the animals have not yet achieved biological balance to respond to stress. Finally, the choice of the adequate type of assay falls on the balance between the advantages and disadvantages of laboratory and *in situ* assays even though both proved adequate for surveying risk of even moderately contaminated sediment by multiple classes of substances. Laboratory assays are likely to enhance bioavailability, therefore unleashing the full risk potential of a sediment and ameliorate the effects on confounding variables. However, *in situ* assays provide a more realistic ecological risk assessment of environmental contamination (although possessing other disadvantages such as cost and response variation driven by environmental factors); therefore gaining for true predictive ecotoxicology whatever information might be lost for more mechanistic studies.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). For their help and support, the authors would like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA); J. Raimundo, V. Branco, R. Cesário and P. Pousão (IPIMAR-INRB); J.P. Sampaio, A. Fonseca and M. Ferreira (DCV, FCT/UNL) and also A. Milhinhos and M. Simões (ITQB).

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## 4.4. Hepatic proteome changes in *Solea senegalensis* exposed to contaminated estuarine sediments: a laboratory and *in situ* survey[†]


### Abstract

Ecological risk assessment of estuarine sediments poses a challenge to ecotoxicologists due to the complex geochemical nature of sediments and to the combination of multiple classes of toxicants. Taking advantage of the proteomic high throughput approach, juvenile Senegalese soles were exposed for 14 days in the laboratory and *in situ* (field) to sediments from three sites (a reference plus two contaminated) of a Portuguese estuary. Sediment characterization confirmed the presence of a mixture of metals, polycyclic aromatic hydrocarbons and organochlorines in the two contaminated sediments. Deregulation of liver cytosolic proteins was determined by a combination of two-dimensional electrophoresis with *de novo* sequencing by tandem mass spectrometry. A total of nineteen deregulated proteins were able to be identified, taking part in multiple cellular processes such as anti--oxidative defence, energy production, proteolysis and contaminant catabolism (especially oxidoreductase enzymes). Besides a clear distinction between animals exposed to the reference and contaminated sediments, differences were also observed between laboratory- and *in situ*-tested fish. Soles exposed in the laboratory to the contaminated sediments failed to induce, or even markedly downregulated, many proteins, with the exception of a peroxiredoxin (an anti-oxidant enzyme) and a few others. *In situ* assays revealed greater protein deregulation in animals exposed to the sediment most contaminated by organic substances. Downregulation of basal metabolism enzymes linked to energy production and gene transcription related to exposure to sediment-bound contaminants and was a very significant consequence of chemical-induced stress that compromised the organisms' ability to deploy adequate response and defence mechanisms against insult.



### Keywords

Proteomics; Flatfish; Bioassays; Ecological risk assessment; Sediment contamination; Sado Estuary


[†] Costa et al. (*submitted*).

## 1. Introduction

High throughput “omic” techniques have been broadly gaining space in almost all fields of research where fish are employed as test subjects, even though fish do not benefit from the same levels of genomic and proteomic sequencing and annotation than other vertebrate model. In fact, the zebrafish (*Danio rerio*) can be considered as the one true “model” fish species, as the rat and mouse are for mammals and *Arabidopsis* for plants. Nevertheless a few other potential candidates have sprung out in literature, like the tilapia or the medaka. Among the three major “omic” approaches (proteomics, metabolomics, transcriptomics), proteomic studies in fish have been applied to multiple areas of biology, from embryogenesis to aquaculture of economically important species. The application of such techniques to environmental toxicology has already begun (see Forné et al., 2010, for a recent review on fish proteomics).

With respect to environmental monitoring, surveying the potential impact of aquatic sediment contamination to organisms has been a considerable challenge to ecotoxicologists. These media constitute important reservoirs for contaminants, with especial respect to estuarine sediments, due to the strong anthropogenic pressure put onto estuaries and the particular physico-chemical nature of transition water bodies. In addition, the deposition and release of contaminants trapped in estuarine sediments is complex and depends on multiple factors such as substance speciation, sediment organic carbon load, grain size, disturbance and shifts in the oxic/anoxic state, just to account for some important abiotic factors (Atkinson et al., 2007). The combination of all these parameters with the most probable presence of multiple classes of contaminants constitutes a substantial confounding factor when assessing either the potential impact of sediment-bound contamination to organisms (for “predictive” ecotoxicology) or surveying the biological machinery of defence and detoxification (“mechanistic” ecotoxicology). In fact nowadays there is even a strive to find adequate non-specific biomarkers in estuarine animals that can be effectively employed in ecotoxicological studies where complex contaminant mixtures are involved (Monserrat et al., 2007). Bringing the biomarker approach to a new, state-of-the-art, molecular level, high throughput “omic” techniques began over the past decade to be employed in eco- and environmental toxicology. Such research has, in most cases, intended to survey patterns of response, potential biomarkers and contribute to the understanding of the very complex intracellular machinery that underlies chemical insult, taking advantage of the screening-based approach that these methods are based on (refer to Monsinjon and Knigge, 2007, for a review).

Either for the purpose of ecological risk assessment (ERA) or for more mechanistic approaches to toxicity assessment, bioassays are the backbone of much research with aquatic organisms. Still, although some authors already reported differences between laboratory and field assays with fish for the purpose of biomonitoring natural sediments (for instance, Vethaak et al., 1996; Hatch and Burton, 1999), little research exists comparing the two types of bioassays and fewer or none reporting on “omic” surveys. Regardless of the methodology to determine the effects and responses to

toxicity in organisms, such comparison has been determined of relevance since field (*in situ*) assays are affected by environmental variables other than contamination, laboratory assays, on the on the other hand, are rarely ecologically realistic and tend to be conservative (Chapman, 2007).

Among the piscine species that have been employed in environmental toxicology studies, flatfishes (Teleostei: Pleuronectiformes) have been receiving growing attention for the environmental monitoring of coastal areas potentially affected by contaminated sediments due to their benthic disposition. Complementarily, studies focusing on more mechanistic approaches using these animals as models with the application of "omics" are just springing out (Cerdá et al., 2010). Species like the flounder (*Platichthys flesus*) have been responsible for a large number of publications in ecotoxicology and have recently met their SW European counterpart, the soleid *Solea senegalensis*. The Senegalese sole, *Solea senegalensis* Kaup, 1858, is a flatfish of important value for fisheries and, especially, for aquaculture in Southern Europe. In its natural habitat, the species inhabits muddy or sandy floors (where it feeds on small invertebrates) of coastal areas, especially estuaries, since they constitute important breeding and nursing grounds (Cabral and Costa, 1999; Cabral, 2000). Much research has been performed on this species in the areas of aquaculture, marine ecology and ecotoxicology. "Omic" (proteomic and transcriptomic) studies have, inclusively, been performed on Senegalese soles, although more for aquaculture- than for ecotoxicology-related studies (e.g. Forné et al., 2009; Salas-Leiton et al., 2009; Osuna- Jiménez et al., 2009; Costa et al., 2010b).

The present work aims at surveying alterations to hepatic cytosolic protein regulation patterns in juvenile Senegalese soles exposed to contaminated sediments collected from an anthropogenic--impacted estuary (The Sado, W Portugal) through a dual arrangement of laboratory and *in situ* bioassays to compare the two assay methodologies. It is intended to survey protein deregulation in an ERA context for the study area and to contribute to the understanding of the biological mechanisms underlying exposure to complex mixtures of contaminants, as occurring in natural sediments.

## 2. Methods and materials

### *2.1. Experimental design and procedure*

The study area (Fig. 4.4.1) consists of a large estuarine basin of high socio-economical importance that has long been subjected to human pressure. The estuary is impacted by many different sources of pollution, both point and diffuse. The area comprises the city of Setúbal (with an important commercial harbour) and adjacent suburban areas, and a large heavy-industry belt that includes chemical plants, a thermoelectrical unit, shipyards, ore deployment facilities, a paper mill and others. The estuary is also very important for tourism, local fisheries and maritime transport. To all these pressures it can be added the runoffs from upstream agriculture grounds that likely carry pesticides and fertilizers, besides being the river itself an important source of metals since it transverses a pyrite

mining region (Cortesão and Vale, 1995). In addition, a large part of the estuary is classified as environmental protection area. Recent surveys found sediment from the estuary to be moderately contaminated by mixed classes of organic and inorganic substances, however, the observed levels of contamination have been found to induce harmful effects on organisms (see for instance Caeiro et al., 2009; Costa et al., 2009, 2010a and references therein).

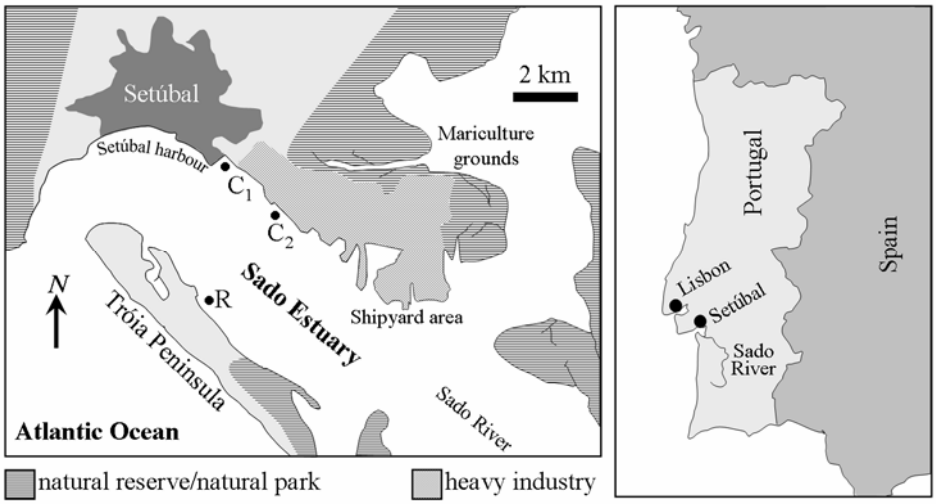


**Fig. 4.4.1.** Location map of the Sado Estuary around the city of Setúbal, showing the sediment collection and *in situ* assay sites (•): R (reference) plus $C_1$ and $C_2$ (contaminated).

Juvenile Senegalese soles (standard length = 61.0 ± 8.4 mm; total wet weight = 3.1 ± 1.6 g) were exposed to sediments from three surveyed sites, R (reference), $C_1$ and $C_2$ (Fig. 4.4.1), through simultaneous laboratory and *in situ* (field) 28-day bioassays. The three sites were chosen according to information obtained from a previous ERA strategy for the estuary (refer to Caeiro et al., 2009). Fish were hatchery-brood and laboratory-reared and were all from the same cohort. The sediments were collected with a grab from each site on May 2007 for physico-chemical characterization and preparation of the laboratory assays. For simplification, exposure to the three sediments will be referred to as tests R, $C_1$ and $C_2$.

The laboratory assays were prepared by placing 2 L of fresh sediments in 15 L-capacity white polyvinyl tanks with blunt edges to which was added 12 L of clean, filtered seawater. A recirculation arrangement was adapted to the test tanks, as well as permanent aeration (dissolved $O_2$ ≈ 55-60%). Water and air flows were adjusted to avoid sediment disturbance. Additionally, the sediments were allowed to settle for 48 h before the beginning of the assays. A weekly 25% water change was done in order to maintain standard water quality (salinity = 32.1 ± 0.3, pH 8.0 ± 0.1 total ammonia = 1.6 ± 0.6 $mg.L^{-1}$, unionized, toxic, ammonia = 0.04 ± 0.02 $mg.L^{-1}$; similar to rearing conditions) with minimal removal of waterborne contaminants and suspended matter. Animals were fed daily with commercial pellets for aquaculture fish. Temperature and photoperiod were set at 18 ± 1 ºC and 12:12 h light:dark, respectively. All tests were performed in duplicate (meaning two tanks per test), with twenty randomly-selected fish being allocated per tank. The *in situ* assays were performed at the location of

sediment collection with submerged cages (consisting of a plastic 90 × 90 × 30 cm frame lined by a 5 mm plastic mesh) placed by scuba diving over the bottom, ensuring direct contact of animals to the sediment. Each cage was divided in two equal-sized compartments regarded as replicates. Each compartment held 20 randomly-selected fish.

Sampling occurred after 14 days of exposure. The animals exposed *in situ* were transported alive to the laboratory where, as laboratory-tested fish, were euthanized by cervical sectioning before dissection and liver excision. Animals collected at "day 0" ($T_0$) consisted of fish collected directly from the rearing tanks.

*2.2. Sediment characterization*

Sediments were analysed for inorganic (element) and organic contaminants from dried samples. The non-metal selenium (Se), the metalloid arsenic (As) and the metals cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn), were quantified by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X--Series equipment, after mineralization with acids (HCl, $HNO_3$ and HF) in closed Teflon vials according to Caetano et al. (2007). Total mercury (Hg) was determined by atomic absorption spectrometry (AAS) according to Costley et al. (2000), after sample pyrolysis of (750 ºC in an oxygen atmosphere) in a combustion tube attached to an AMA-254 mercury analyzer (Leco). The same protocols were applied to the certified reference sediments MESS-2and PACS-2 (National Research Council, Canada) plus MAG-1 (US Geological Survey, USA) to verify the adequateness of the procedure and the measurements were found within the certified range.

Sediment PAHs (comprising a total of seventeen 3- to 6-ring compounds) were quantified by gas chromatography-mass spectrometry (GC-MS) after Soxhlet extraction with an acetone+hexane (1:1 v/v) mixture (Martins et al., 2008). Organochlorines, namely eighteen polychlorinated biphenyl (PCB) congeners and the dichloro diphenyl thichloroethane (DDT) pesticide (with total DDT meaning the sum of concentrations of *pp*'DDT plus its main metabolites *pp*'DDE and *pp*'DDD) were determined by GC with electron capture detection (GC-ECD) after Soxhlet extraction with *n*-hexane and column fractioning (Ferreira et al., 2003). The procedure was validated by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the obtained values were found within the certified range.

The sediments' redox potential (Eh) was measured immediately after collection with an electrode apparatus equipped with a platinum electrode with an Ag/AgCl reference electrode. Sediment total organic matter (TOM) was extrapolated from total organic carbon loss-on-ignition after combustion at 500 ºC for 5 h. Fine particle fraction (FF), particle size < 63 μm, was determined after disaggregation with pyrophosphate and hydraulic sieving.

*2.3. Proteomic analysis*

The alteration to hepatic cytosolic proteins' expression pattern was determined by two--dimensional electrophoresis (2DE) as developed by Romero-Ruiz et al. (2006) and Montes-Nieto et al. (2007) and described by Salas-Leiton et al. (2009) and Costa et al. (2010b) for the species, with modifications. For each experimental condition (exposure either in laboratory or in the field to the three sediments, plus $T_0$ animals), frozen liver samples from four randomly selected individuals per experimental condition (two per replica) were pooled and homogenized in liquid nitrogen. Crude protein extraction was achieved by placing ≈ 100 mg from each liver homogenate in 20 mM Tris-HCl buffer (pH 7.6) with 0.5 M sucrose and 0.15 M KCl and complemented with 60 $\mu L.ml^{-1}$ 2--hydroxyethyl disulfide (HED) as reducing agent. The HED agent was employed after preliminary trials that showed it to very significantly reduce streaking in second-dimension gels when compared to the more commonly used dithiothreitol (DTT). One mM phenylmethylsulfonyl fluoride (PMSF), 6 mM leupeptin and 100 $\mu L.mL^{-1}$ of Protease Inhibitor Cocktail (Sigma) were added to the extraction buffer as protease inhibitors. After grinding with a pestle, samples were centrifuged for 1 min to remove the lipid supernatant, followed by another 10 min centrifugation (14,000 × g at 4 ºC). Samples were then treated with 500 $U.mL^{-1}$ benzonase endonuclease for 30 min (at room temperature), followed by centrifuging at 102,000 × g for 1 h (at 4 ºC) to precipitate the remaining non-peptide material. Total protein was determined according to Bradford (1976) so each immobilized pH gradient (IPG) strip (18 cm, pH 4-7; from GE Healthcare) could be loaded with 100 μg of protein. Three IPG strips were prepared per each experimental condition, meaning all subsequent steps were performed in triplicate. Strips were incubated (30 min at room temperature) in pH 4-7 IPG buffer (GE Healthcare) complemented with 7 M urea, 2% w/v of the non-ionic detergent CHAPS, 60 $\mu L.mL^{-1}$ of the HED reducing agent, plus ≈ 1% w/v bromphenol blue. Strips were then allowed to passively rehydrate (6 h, 20 ºC) and afterward subjected to isoelectric focusing (IEF) on a Protean IEF apparatus (Bio-Rad) for first-dimension protein separation (according to isoelectric point). The second dimension separation (by molecular weight) was achieved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 18 × 18 cm 12.5% acrylamide/bis-acrylamide gels. Prior, IPG strips were equilibrated in 1.5 M Tris-HCl buffer (pH 8.8) to which were added 6 M urea and 2% SDS as denaturing agents; 60 $\mu L.mL^{-1}$ HED, 3% glycerol and approximately 1% w/v bromphenol blue [equilibration of IPG strips was followed by a treatment with 25 mM iodacetamide (IAA) to block sulphydryl groups]. Electrophoresis was then run at constant wattage (10 W per gel) in a DodecaCell Plus device (Bio--Rad). The SigmaMarker wide-range protein ladder (Sigma-Aldrich) was used as molecular weight (MW) standard. Gels were afterwards stained with the Sypro Ruby fluorescent dye for peptides (Bio--Rad) and imaged with a Bio-Rad FXImager laser scanner. All image analyses and determination of protein regulation factors were done with ProteomWeaver (Bio-Rad).

The protein spots in gels were selected for identification according to the criteria of a significant statistical difference in spot intensity (given by the Mann-Whitney *U* test) between at least one experimental test to $T_0$ gels (used as the calibrator group for statistical purposes) plus a minimum

of 50% up- or downregulation to $T_0$ condition, to ensure contrast between treatments (e.g. Montes--Nieto et al., 2007; Costa et al., 2010b). The spots that met the criteria were excised from gels treated with DTT and IAA prior to digestion with porcine trypsin. The digestion products were then preliminarily sequenced by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in a Voyager De-PRO apparatus (Applied Biosystems) to determine the quality of the samples and eliminate background from trypsin self-proteolysis. The samples were afterwards subjected to *de novo* sequencing by capillary liquid chromatography electrospray ionization ion trap tandem mass spectrometry (capLC-ESI-ITMS/MS) using a LTQ system (ThermoFisher Scientific). Peptide search was performed using the Protein-Protein Blast 2.2.20 software (Altschul et al., 1997) to contrast results to chordate, actinopterygian and pleuronectiform taxa peptide sequences existing in the NCBI (National Centre for Biotechnology Information of the USA) All-Non Redundant (nr) Protein Sequence database. The results were validated according to the criteria of highest score and lowest *e*-value and also to number of matched peptides and representation in the prospected database sets.

### *2.4. Statistical analysis*

The statistical significance of the differences between spot intensities were surveyed by the non-parametric Mann-Whitney *U* test considering the small *n* ($n$ = 3) for parametric analyses. Statistics were performed using the software Statistica (Statsoft Inc.). A significance level of $\alpha = 0.05$ was set for all analyses. The identified proteins' relative expression was assessed as the percentage of spot intensity relatively to $T_0$ fish. Cluster analysis on protein expression data was performed with the software DAnTE (Polpitiya et al., 2008).

## 3. Results

### *3.1. Sediment characterization*

As shown in figure 4.4.2, the reference sediment (sediment R) was confirmed to be the least contaminated, as well as the least anoxic (Eh ≈ -140 mV) and the most sandy (FF = 2%, TOM = 23%). The two most contaminated sediments revealed distinct patterns of contamination; although both contained a blend of organic and inorganic xenobiotics. Sediment $C_1$ (located closest to the city of Setúbal, Eh ≈ -300 mV, FF = 96 %, TOM = 10 %) was the most contaminated by metals while sediment $C_2$ (located off the city's heavy industry area, Eh ≈ -300 mV, FF = 76%, TOM = 7%) was observed to be the most contaminated by PAHs and organochlorines even though the levels of sediment organic contaminants were very similar between $C_1$ and $C_2$ (Fig. 4.4.2). In general, these results are in accordance with previous characterization of the sites (Costa et al., 2009), still site $C_2$

revealed an increase in metal and organochlorine concentrations. Still, by comparison with previous research in the area, the sediments can be globally regarded as moderately contaminated (Costa et al., 2010a).

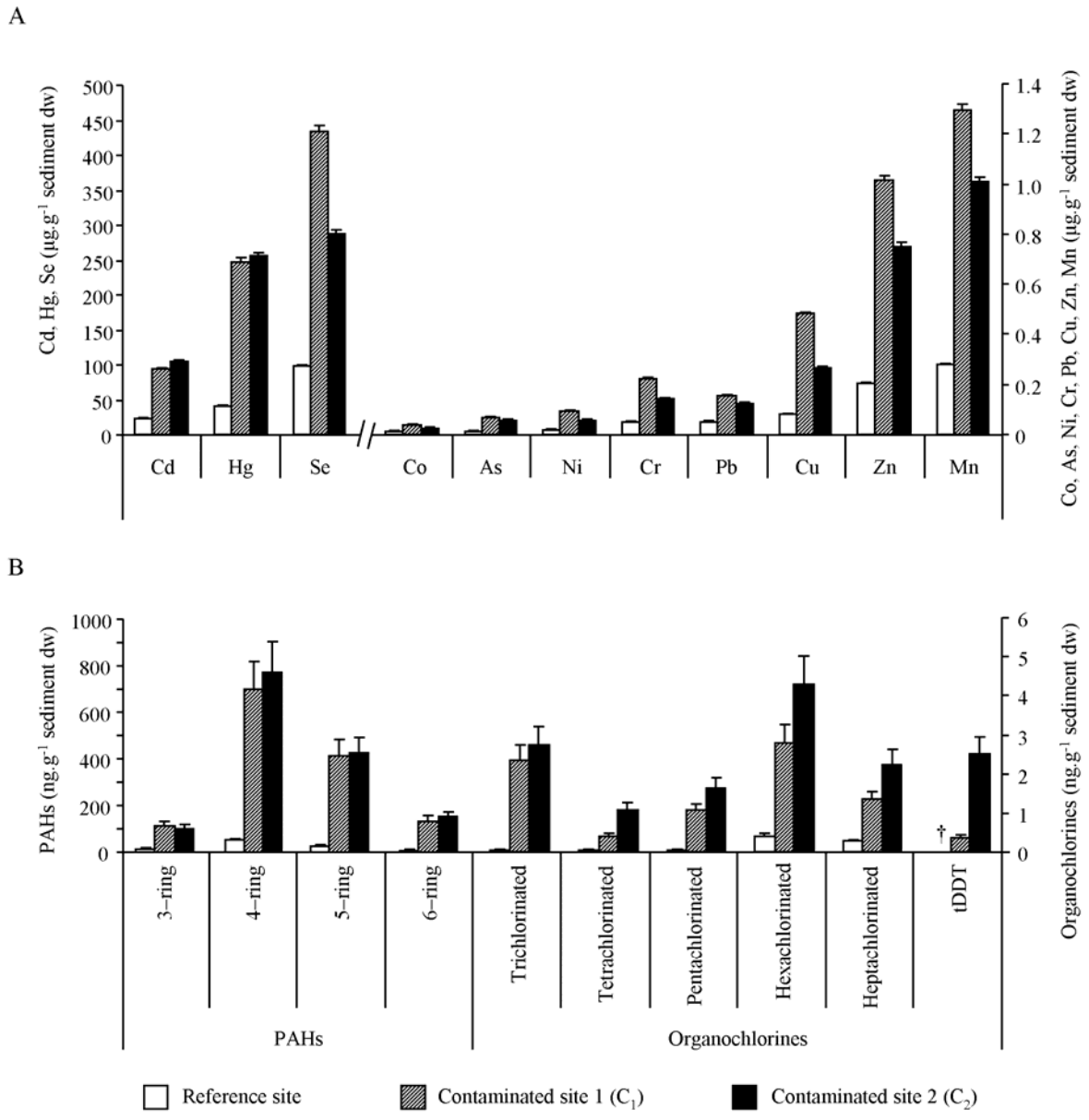


**Fig. 4.4.2.** Concentrations of element (A) and organic contaminants (B) in the surveyed sediments R (reference), $C_1$ (most contaminated by metals) and $C_2$ (most contaminated by organic substances. † means below detection limit. The level of Hg in the sediments refers to total Hg (organic plus inorganic species).

Besides the levels of As, Cd and total Hg, similar between the two contaminated sediments, the surveyed elements' concentrations were consistently higher in sediment $C_1$ (Fig. 4.4.2A). The most noticeable differences between $C_1$ and $C_2$ were obtained for Cu (≈ 1.8 fold) and the non-essential Cr and Ni (≈ 1.6 fold for both). Compared to the levels in the reference sediment (R), Cu and Hg were higher in contaminated sediments by approximately 6-fold. Four- and five-ring compounds represented the largest majority of PAHs in all sediments, representing ≈ 75-80% of total PAH

concentrations (Fig. 4.4.2B). Fluoranthene and pyrene had the highest concentrations, with 315.7 and 263.2 ng.g$^{-1}$ sediment dw in sediment $C_1$ and 345.2 and 286.3 ng.g$^{-1}$ sediment dw for $C_2$, respectively. For all sites, the phenanthrene/anthracene and fluoranthene/pyrene ratios were > 1 and < 10, respectively, indicating mostly pyrolytic origin (combustion-derived) of PAHs rather than petrogenic [i.e. derived from fossil fuels (Budzinski et al., 1997)]. Hexachlorinated PCBs were the most representative PCBs in all sediments, ranging between ≈ 35-50% of total PCB concentrations (Fig. 4.4.2B). The highest concentration for the class was found for PCB 153 in sediment $C_2$ (1.23 ng.g$^{-1}$ from a total of 4.29 ng.g$^{-1}$ sediment dw for hexachlorinated PCBs). Still, the most concentrated PCB was PCB 26, in both $C_1$ and $C_2$ sediments, with 1.8 and 2.0 ng.g$^{-1}$ sediment dw, respectively (not detected in the reference sediment).

*3.2. Proteomic analyses*

The 2DE analyses yielded forty-one cytosolic proteins spots that met the criteria mentioned in a previous sections, from which nineteen proteins could be positively identified by contrasting the ESI-ITMS/MS results to nrNCBI database after quality checking by MALDI-TOF MS (Fig. 4.4.3). In addition, all peptides potentially resulting from keratin contamination were excluded. Due to few sequencing of the species' genome and peptidome, only two proteins could be directly matched to *S. senegalensis*, namely β actin and the trypsin precursor trypsinogen 1c. Still, peptide matching yielded low *e*-values, ranging between the scales of $10^{-6}$ to $10^{-20}$. The best matched protein was the proteasome subunit β type 9with a score of 94.9 and an *e*-value of $6 \times 10^{-20}$ (Table 4.4.1). The identified proteins take part in multiple cellular processes, from cytoskeleton (β-actin), oxidative stress response (like 1-*cys* peroxiredoxin) to enzymes involved in the glycolytic pathway (such as enolases and lactate dehydrogenase), specialized metabolism (glutamine synthetase and glycine-*N*-methyltransferase), proteolysis (cathepsin, trypsinogen as a precursor for trypsin and proteasome subunits) and xenobiotic catabolism for some oxidoreductase enzymes (Table 4.4.2).

Only glutamine synthetase and trypsinogen 1c were found downregulated (below the 50% threshold of expression relatively to $T_0$ fish), in laboratory exposure to sediment $C_1$ and all field tests, respectively. All other cases represent peptides where regulation was either found to be unchanged or increased (above the 150% threshold comparatively to $T_0$ animals). The highest upregulation was obtained for trypsinogen (378%) in $C_1$-tested fish in the laboratory, followed by the Transcription initiation factor IIE subunit β in laboratory assayed fish exposed to the reference sediment, R (298%). Laboratory test with sediments $C_1$ and $C_2$ result in a reduced number of proteins that had their regulation patterns changed compared to all field-assayed animals and animals exposed to sediment R in either type of assay.

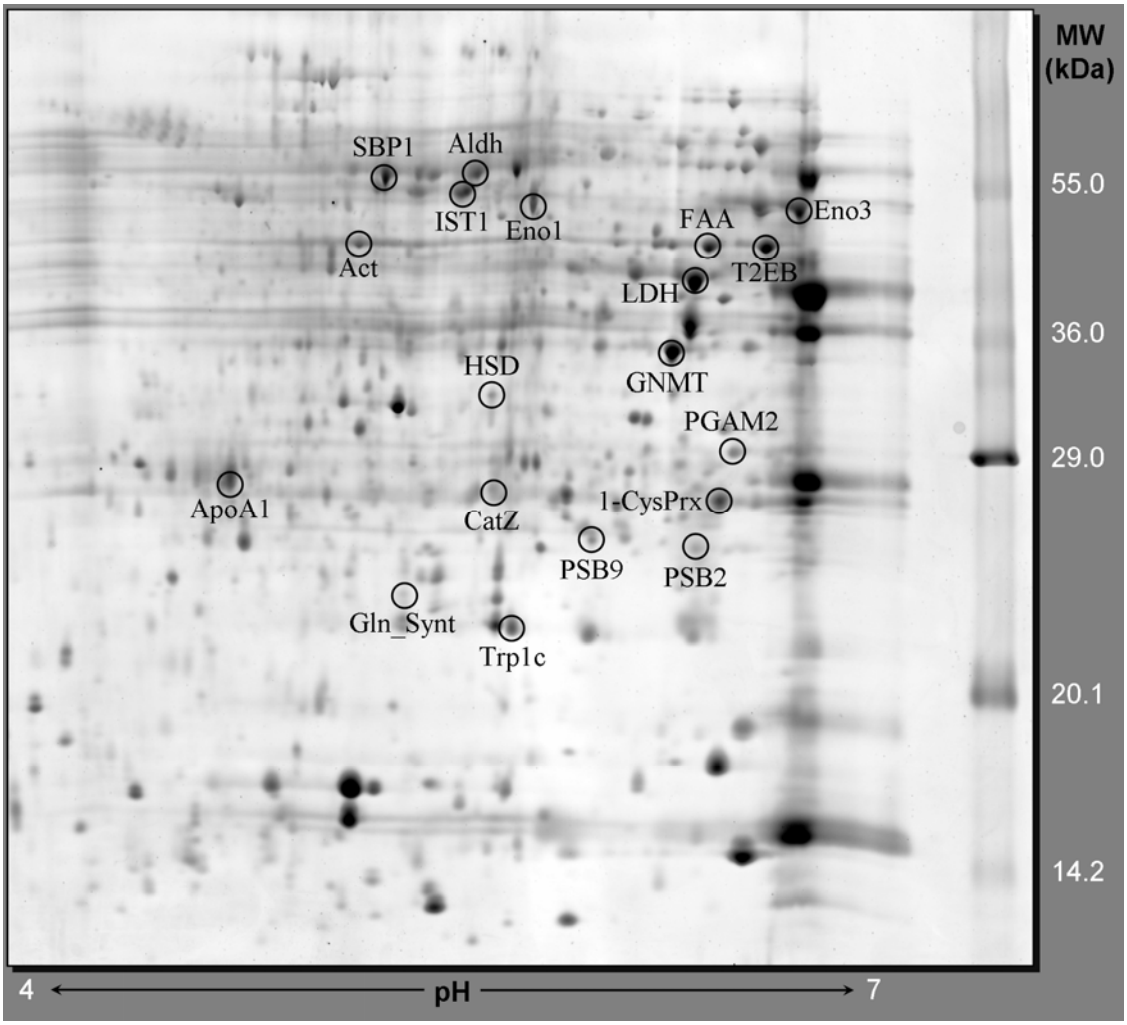


**Fig. 4.4.3.** Representative second dimension SDS-PAGE gel image showing the differentially expressed spots that were identified by ITMS/MS.

Comparing the regulation patterns of $C_1$- and $C_2$-tested fish in either type of assay to the respective reference test (exposure to the clean sediment R in the laboratory or *in situ*), it was observed that only peroxiredoxin and trypsinogen were significantly upregulated in laboratory tested fish ($C_1$ and $C_2$ tests), as well as lactate dehydrogenase (in test $C_1$ only). Regarding the *in situ* experiment, upregulation was observed for aldehyde dehydrogenase, enolase 3, fumarylacetoacetase, glutamine synthetase and lactate dehydrogenase, mostly linked to energy production (via the glycolytic pathway) and aminoacid metabolism processes. Exposure to sediment $C_1$ (most metal-contaminated) in the laboratory resulted in the greatest percentage of downregulated proteins compared to the reference test, with a total of, twelve out of nineteen proteins being underexpressed, followed by the $C_2$ laboratory test, with seven (Fig. 4.4.4).

**Table 4.4.1.** Cytosolic protein identification summary after *de novo* sequencing using ESI-ITMS/MS and peptide sequence database search with Protein-Protein Blast and correspondent regulation factors (percentage of relative regulation ± standard deviation) after the 14-day exposure to the reference (R) and contaminated sediments ($C_1$ and $C_2$). Regulation factors were estimated taking $T_0$ fish (calibrator group) as baseline reference. Boldface numbers indicate significant changes to regulation patterns (over $T_0$ fish) according to the imposed 50% up- or -downregulation threshold.

| | | | | | | | Protein regulation factor | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Laboratory assay | | | *In situ* assay | | |
| **Protein** | Abbreviation | Uniprot Accession | Database taxa[a] | Score | *e*-value | Matched peptides | R | $C_1$ | $C_2$ | R | $C_1$ | $C_2$ |
| 17-beta hydroxysteroid dehydrogenase | HSD | Q7T2J0 | 1,2,3 | 83.3 | $4 \times 10^{-17}$ | 4 | **203.2 ± 14.2** | 138.9 ± 18.1 | 144.9 ± 6.6 | **239.3 ± 8.9** | **259.5 ± 14.2** | **178.1 ± 11.2** |
| 1-*Cys* peroxiredoxin | 1-CysPrx | Q8AWH6 | 1,2,3 | 41.8 | $1 \times 10^{-6}$ | 2 | 71.7 ± 7.1 | 127.7 ± 27.6 | **150.6 ± 19.3** | 89.1 ± 18.5 | 120.1 ± 11.9 | 77.8 ± 17.2 |
| Aldehyde dehydrogenase | Aldh | Q98TM9 | 1,2,3 | 63 | $6 \times 10^{-13}$ | 5 | **203.4 ± 4.9** | 106.9 ± 10.3 | 101.6 ± 5.9 | **163.7 ± 20.2** | **163.0 ± 20.3** | **211.0 ± 8.8** |
| Apolipoprotein A-1 | ApoA1 | O42363 | 1,2,3 | 60 | $4 \times 10^{-10}$ | 3 | 139.2 ± 9.1 | 136.2 ± 15.2 | 113.9 ± 15.5 | **158.0 ± 18.7** | **154.6 ± 7.5** | 90.0 ± 11.3 |
| β Actin | Act | Q1HHC7 | 1,2,3 | 71 | $2 \times 10^{-15}$ | 8 | 139.1 ± 12.8 | 95.8 ± 6.5 | 119.1 ± 13.8 | **225.3 ± 15.3** | 140.6 ± 16.6 | **159.3 ± 6.6** |
| Biphosphoglycerate mutase 2 | PGAM2 | B5XD74 | 2,3 | 52 | $1 \times 10^{-7}$ | 3 | **161.8 ± 11.4** | 108.6 ± 3.0 | 129.4 ± 25.2 | 103.7 ± 9.2 | 105.8 ± 15.2 | 87.9 ± 19.4 |
| Cathepsin Z | CatZ | Q58HF4 | 1,2,3 | 77 | $8 \times 10^{-15}$ | 1 | 117.1 ± 4.9 | 99.5 ± 23.2 | 101.8 ± 55.1 | **167.3 ± 27.5** | 141.9 ± 17.5 | **196.0 ± 10.4** |
| Enolase 1 | Eno1 | C0H878 | 2,3 | 57.5 | $3 \times 10^{-9}$ | 4 | 139.2 ± 9.1 | 136.2 ± 15.2 | 113.9 ± 15.5 | **158.0 ± 18.7** | **154.6 ± 7.5** | 90.0 ± 11.3 |
| Enolase 3 | Eno3 | Q568G3 | 2,3 | 68.1 | $2 \times 10^{-12}$ | 1 | **165.7 ± 6.1** | 80.7 ± 16.0 | 72.7 ± 29.7 | 127.8 ± 8.2 | 150.7 ± 6.5 | 147.7 ± 20.2 |
| Fumarylacetoacetase | FAA | Q803S0 | 2,3 | 46 | $7 \times 10^{-6}$ | 1 | **193.9 ± 2.3** | 120.9 ± 2.8 | 108.7 ± 6.3 | 97.8 ± 13.9 | 122.2 ± 14.0 | **193.4 ± 4.9** |
| Glutamine synthetase | Gln_Synt | Q4RVF3 | 1,2,3 | 72.4 | $2 \times 10^{-13}$ | 2 | 85.9 ± 19.5 | **47.6 ± 23.2** | 63.9 ± 21.8 | 120.5 ± 1.6 | 97.2 ± 6.7 | 84.4 ± 8.5 |
| Glycine *N*-methyltransferase | GNMT | Q6P607 | 2,3 | 71 | $2 \times 10^{-13}$ | 4 | 140.2 ± 5.8 | 129.7 ± 8.1 | 130.3 ± 6.2 | 122.4 ± 3.2 | 137.3 ± 6.6 | **161.7 ± 9.1** |
| Malate/*L*-lactate dehydrogenase | LDH | Q7T3D9 | 2,3 | 75.3 | $1 \times 10^{-14}$ | 2 | 129.7 ± 8.7 | **168.2 ± 17.8** | 128.6 ± 15.3 | **158.0 ± 16.0** | **181.7 ± 7.1** | **152.3 ± 16.0** |
| MAPK activating protein PM28 | IST1 | P53990 | 2,3 | 76.3 | $2 \times 10^{-14}$ | 3 | 149.0 ± 9.8 | **154.6 ± 27.1** | 125.8 ± 16.5 | **203.3 ± 10.6** | 139.2 ± 10.2 | **214.4 ± 13.4** |
| Proteasome subunit β type 2 | PSB2 | Q6DHI9 | 2,3 | 52.4 | $9 \times 10^{-8}$ | 2 | **244.8 ± 5.5** | 144.8 ± 11.9 | 121.9 ± 16.3 | **231.9 ± 14.8** | **221.9 ± 17.5** | **236.2 ± 11.8** |
| Proteasome subunit β type 9 | PSB9 | Q9DD33 | 1,2,3 | 94.9 | $6 \times 10^{-20}$ | 2 | 126.5 ± 7.9 | 79.9 ± 23.4 | 94.8 ± 32.6 | **155.3 ± 7.2** | **162.4 ± 13.9** | 114.7 ± 9.0 |
| Selenium binding protein 1 | SBP1 | Q6PHD9 | 2,3 | 69.7 | $1 \times 10^{-12}$ | 3 | **272.7 ± 10.9** | **195.3 ± 18.9** | **216.0 ± 13.7** | **234.5 ± 6.1** | **230.2 ± 1.8** | 127.6 ± 26.0 |
| Transcription initiation factor IIE subunit β | T2EB | T2EB | 2,3 | 56.6 | $5 \times 10^{-9}$ | 2 | **298.2 ± 9.1** | 126.0 ± 6.2 | 137.6 ± 20.6 | **228.8 ± 5.1** | **265.4 ± 18.2** | **205.1 ± 8.3** |
| Trypsinogen 1c | Trp1c | A7VMR6 | 1,2,3 | 54.5 | $2 \times 10^{-10}$ | 2 | 91.8 ± 9.0 | **378.0 ± 8.1** | **165.8 ± 4.9** | **48.0 ± 5.9** | **23.8 ± 36.9** | -[b] |

[a]nrNCBI database taxa from which peptide matched was obtained: 1-Order Pleuronectiformes; 2-Class Actinopterygii; 3-Phylum Chordata. [b]no expression was observed in any of the triplicate gels of the experimental condition.

**Table 4.4.2.** Main function of the identified proteins.

| Function class | Protein | Activity |
|---|---|---|
| *Aminoacid metabolism* | Fumarylacetoacetase | Metabolism of aromatic aminoacids. |
| | Glutamine synthetase | Glutamine biosynthesis (catalyses the ammonia-glutamate bond). |
| | Glycine *N*-methyltransferase | Methionine metabolism. |
| *Cell cycle progression* | MAPK activating protein PM28 | Involved in cell division, probably in cytokynesis. |
| *Cell structure* | β actin | Key component of the cytoskeleton. |
| *Energy production* | Biphosphoglycerate mutase 2 | Glycolysis. |
| | Enolase 1 and 3 | Glycolysis and gluconeogenesis. |
| | Malate/*L*-lactate dehydrogenase | Involved in anaerobic energy production (fermentation). |
| *Gene transcription* | Transcription initiation factor IIE subunit β | Part of the RNA polymerase initiation complex. |
| *Intracellular signalling* | Selenium binding protein 1 | Unknown. May be involved in xenobiotic sensing in cytoplasm and in anti-carcinogenic mechanisms. |
| *Lipid transport* | Apolipoprotein A-1 | Promotes cholesterol efflux from cells. |
| *Oxidoreductase enzymes* | 1-*Cys* peroxiredoxin | Scavenges oxidative radicals. Evidence for function in phospholipid turnover. |
| | 17-beta hydroxysteroid dehydrogenase | Androgen and oestrogen metabolism and biosynthesis. |
| | Aldehyde dehydrogenase | NAD-dependent oxidant catalyst. |
| *Proteolysis* | Trypsinogen 1c | Serine protease (protease S1 family). |
| | Cathepsin Z | Cysteine protease (protease C1 family). |
| | Proteasome subunits (β) types 2 and 9 | Component of the proteasome (macropain) multicatalytic proteinase complex (ATP-dependent). |

## 4. Discussion

Chronic exposure to toxicants may induce defences against chemical insult or impair specific responses depending on time of exposure, contaminant concentrations, the existence of a combination of contaminants (and other factors like exposure route, assay type, etc.), to which can also be added intrinsic biological factors such as metabolic differences between organs and tissue types. In particular, exposure to moderate levels of contamination by multiple xenobiotics has been found to produce results that may contradict expected biomarker responses even though capable of inducing severe chronic lesions (Triebskorn et al., 1997; Mouneyrac et al., 2002; Costa et al., 2009, 2010a). In addition, moderate contamination may yield responses in fish that appear to be less correlated with each other than more severe environmental contaminant profiles (Triebskorn et al., 1997). The present findings indicate that the conjunction of moderate sediment contamination, toxicant mixtures plus the complex geochemical nature of natural sediments can trigger complex patterns of proteome changes in fish liver, to which is added a clear distinction between laboratory and field bioassays.

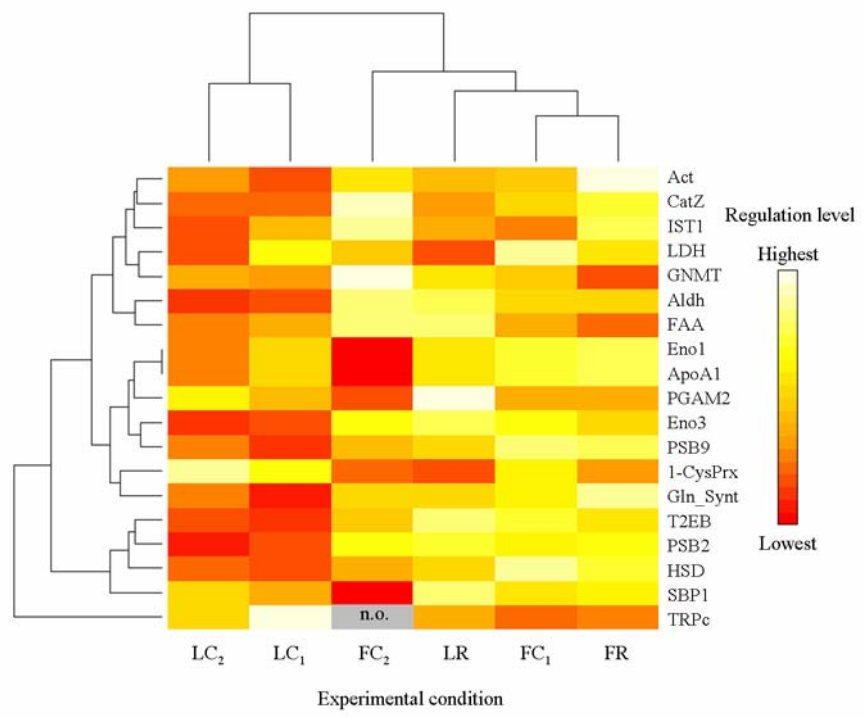


**Fig. 4.4.4.** Cluster analysis on protein regulation factors and experimental tests. Complete linkage was employed as amalgamation rule and Euclidean distances as metrics. [n.o.] means no expression observed. L and F indicate laboratory and *in situ* (field) exposure to sediments R (reference), $C_1$ and $C_2$ (contaminated).

When compared to initial state soles ($T_0$ fish), all tests, including exposure to the reference sediment, elicited some degree of hepatic proteome changes (Table 4.4.1). However, the results from exposure to the clean sediment (from site R) should represent a baseline level of metabolic disturbance caused by the bioassays *per se* (e.g. from having changed the animals' environment). When the proteomic responses of animals exposed to contaminated sediments are contrasted to R-tested ones, an

altered pattern of regulation is revealed that can be explained by differences in the sediments' contamination levels. The differences between laboratory and field assayed animals, on the other hand, should be, in great part, explained by the differential bioavailability of sediment xenobiotics.

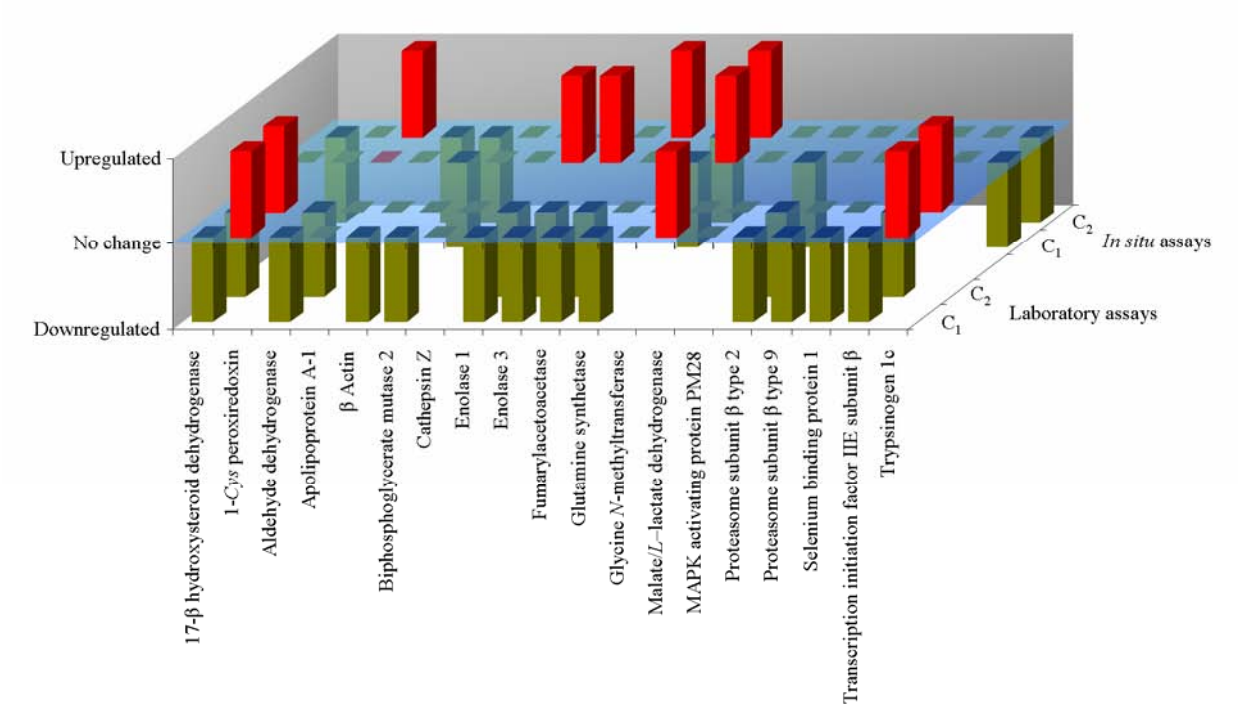


**Fig. 4.4.5.** Altered protein regulation patterns in $C_1$- and $C_2$-tested fish, relative to respective reference test (exposure to sediment R). Columns indicate significantly up- or downregulated proteins (Mann-Whitney *U*, $p < 0.05$).

In previous laboratory bioassays we have already discussed the hypothesis of increased bioavailability of contaminants as consequence of sediment disturbance (caused by collection, handling and animal activity - see, for instance, Costa et al., 2009, 2010a). In fact, aquatic sediments are very complicated matrices of organic and inorganic particles that sorb both hydrophilic and apolar substances. The bond between these substances and sediment particles may be unstable, depending on grain size, redox status and organic matter load and type. In fact, disturbance can greatly alter the sediments steady-state, e.g., by exposing to oxygen the anoxic layers where contaminants are trapped, favouring their desorption (Eggleton and Thomas, 2004). It has also been described the differential release of contaminants, especially organic, for being the most insoluble and therefore displaying a more intricate geochemical behaviour, according not only to molecule type but also age and type of the sediment's organic matter (Kukknonen et al., 2003). Metals, on the other hand, tend to complex with other mineral substances and their release to water (the most effectively bioavailable fraction) may be greatly affected by pH and redox status changes (see for instance Geffard et al., 2005). Thus it is likely that inorganic and organic substances were differentially released from the sediments, a phenomenon that should be of greater relevance in the field, where disturbance was probably less of an issue. It should also not be disregarded that the caged fish (in the field assays) were scavenging the sediments for preys that may themselves accumulate contaminants. This way, ingestion can have taken

its role in exposure whereas in the laboratory assays (where the fish were fed with commercial pellets) the main exposure route was likely via adsorption through gills. The differential availability of xenobiotics can aid explaining why the laboratory-tested fish exhibited more downregulated proteins. On the other hand, while sediments $C_1$ and $C_2$ caused a quite similar proteomic response in the laboratory assays, there were differences in regulation patterns between the two tests *in situ* (Fig. 4.4.5).

*4.1. Differentially-regulated protein patterns may indicate differential bioavailability of toxicants*

By clustering proteins and experimental conditions (Fig. 4.4.5), it can be observed that laboratory exposure to the two most contaminated sediments caused a comparative downregulation of the identified proteins, with the exception of 1-cysPrx (a peroxiredoxin) and TRPc (a trypsin peptidase precursor), when compared to all other tests, including exposure to the same sediments in the field. Although the regulation of these proteins has not been found linked across the experimental conditions, likely due to its unrelated pattern in field-exposed fish, there is a similar response in laboratory-exposed soles to the two most contaminated sediments. Peroxiredoxins are proteins mainly involved in the scavenging of oxidative radicals in the presence of thioredoxins, with evidence for other functions such as phospholipid catabolism, by containing two distinct active sites, one similar to the glutathione peroxidase active site and another similar to a $Ca^{2+}$-independent phospholipase (Chen et al., 2000), which might contribute to its known role in the protection of membrane integrity (Manevich et al., 2002). In addition, peroxiredoxins are regarded as important anti-oxidant enzymes that can be induced by various stressors. There is evidence, at the transcript and protein levels, that the toxic metal Cd induces peroxiredoxins in flatfish liver (Sheader et al., 2006; Costa et al., 2010b). Although David and co-workers (2007) also related a peroxiredoxin gene overexpression to increased estuarine pollution in oysters (in this case, the proteins' isoform 6), there is surprisingly little information relating this family of proteins to environmental contamination.

Trypsinogen (a zymogen), on its turn, is the inactive translation product of trypsin, a serine protease that, besides its role in digestion, has been found linked, either by suppression or induction, to a number of disorders. Although the exact mechanisms and biological meaning of trypsinogen regulation changes in face of toxicological challenge are still unclear, some authors reported that trypsinogen gene expression has some potential as a biomarker for toxicant-induced stress in fish due to its conspicuous underexpression as a consequence of exposure to an organophosphorous pesticide (Sinha et al., 2010). Also, some evidence exists that exposure to Cd and to the combination of Cd plus the PAH benzo[a]pyrene (B[a]P) in *S. senegalensis* induce trypsinogen, but not B[a]P alone, a pattern that was also observed for 1-cysPrx (Costa et al., 2010b). The combination of all this information appears to indicate that in the laboratory studies both metallic and organic toxicants were released (as a result of increased bioavailability), whereas organic contaminants exerted the most compromising effects *in situ*.

Sustaining the hypothesis of the differential bioavailability of organic and metallic contaminants lays the regulation pattern of Glycine *N*-methyl transferase (GNMT), whose overexpression has been found to reduce B[a]P cytotoxicity in human cell-based bioassays (Lee et al., 2006). Other authors found GNMT transcription to be elevated as a consequence of exposure to B[a]P in *Fundulus heteroclitus* embryos, discussing the role of GNMT as a defence strategy due to its role in DNA methylation (Fang et al., 2010). Accordingly, this enzyme was most upregulated in field exposure to sediment $C_2$ (the most contaminated by organic substances, especially PAHs), and significantly upregulated for both $C_1$ and $C_2$ relatively to the reference test, which was not observed in the laboratory assays.

*4.2. Exposure to sediment-bound mixtures of contaminants impaired responses to toxicity - evidence for inhibited gene transcription*

Little information exists regarding direct action of toxicants in the inhibition of gene transcription *per se*. The downregulation of the transcription initiation factor T2EB (also termed transcription factor IIE, TIF IIE subunit β) in fish exposed to the contaminated sediments in the laboratory, compared to $T_0$ and also to reference-tested fish, can contribute to explain the overall downregulation of protein responses observed in fish subjected to this test. Some authors reported microarray data showing that genes related to transcription control can be affected, for instance, by exposure to oxidative substances (Weigel et al., 2002). Also, a significant upregulation of ATF7, a leucine zipper (bZIP) transcription factor, has been recently reported in metal-polluted sites exposed to oxidative stress (Montes-Nieto et al., 2010). In spite of the importance of TIF IIE to the RNA polymerase II initiation complex (Peterson et al., 1991), no information was found regarding the interference of toxicants on its regulation. It is plausible though, that the impairment of gene translation by affecting the regulation of this important protein significantly affected, besides general metabolic processes but also some of the crucial response and defence mechanisms towards exposure to toxicants in fish exposed to sediments $C_1$ and $C_2$. Among such responses there can be found the little studied SBP1, for instance.

The function and mechanism of action of selenium-binding proteins (SBPs) are not yet fully understood. These ≈ 54 kDa proteins have been discovered to be induced by organic contaminants such as PAHs (Ishida et al., 1998) and organochlorines (Ishii et al., 1996). It is therefore not clear why this protein appeared downregulated as a consequence of exposure to sediment $C_1$, in the laboratory, comparatively to the reference test. Interestingly, downregulation of SBP1 has been found linked to carcinogenesis in mammal models and it is suspected that this protein mediated ROS (reactive oxygen species) -induced apoptosis of neoplasic or pre-neoplasic cells (refer, for instance to Pohl et al. (2009). In accordance, some evidence exists towards SBP1 downregulation by oxidative stress (Giometti et al., 2000). Altogether, it is likely that SBP1 has some role in the cell and tissue defence mechanism against injury. Its downregulation may thus have compromised liver responses to insult in fish

exposed to contaminated sediments.

Tissue clean-up of damaged cells (e.g. through apoptosis) and cell proliferation can be an important part of organ recovery following toxicant-induced stress. Some proteins taking part in these functions have been found closely linked (see Fig. 4.4.5), namely the structural protein β actin, IST1 (linked to cell cycle progression) and a cathepsin cysteine protease, CatZ, the latest belonging to a class that is known to intervene in programmed cell death (Chwieralski et al., 2006). The more pronounced downregulation of such proteins in laboratory exposure to sediments $C_1$ and $C_2$ is yet another evidence of stress-response impairment that can be related to increased xenobiotic bioavailability during the laboratory assays. It should be noted, though, that CatZ and IST1 were the only proteins whose regulation in fish exposed to contaminated sediments did not exhibit any significant variation above or below the 50% threshold compared to the reference test in either type of bioassay. It is thus possible that deregulation of these enzymes is partly linked to experimental stress.

The proteasome is an ATP-dependent proteolytic complex that can also be important in eliminating cytoplasmic proteins marked for destruction (by ubiquitination) and therefore have an important regulatory process after injury or changes in its metabolism after chemical-induced stress. It should be noted, though, that other peptidases, as the aforementioned trypsin, also have an important role in cellular and tissue clean-up after damage although the functions and energy demands are dissimilar. Serine proteases as trypsin, for instance, are also suspected to have an important role in apoptosis (Stenson-Cox et al., 2003). Nevertheless, trypsinogen regulation was affected in a different manner by exposure to the contaminated sediments than the proteasome subunits, with no relation being found between these two very distinct (in function, structure and regulation) proteases. Impairment of the proteasome activity has been linked to a series of disorders in humans and model animals, from neurodegeneration (Bedford et al., 2008) to some forms of hepatocellular degeneration, such as the keratin aggresome-based Mallory-Denk bodies (Harada et al., 2008). Even though research on proteasome induction and activity is currently under a spotlight for its potential application in anti--cancer treatments, little specific information exists regarding the effects of toxicants. Nevertheless, Biales et al. (2011), for instance, discovered proteasome subunit downregulation in a proteomic survey in fish exposed to pesticides and their mixtures and Bardag-Gorce and co-workers (2006) found activity and regulation of proteasome to be downregulated in human cultured liver cells exposed to ethanol as a consequence of ROS production, which is consistent with the present findings even though it is not clear why PSB 2 and 9 relative regulation appeared to be little correlated (Fig. 4.4.5). Still, proteasome subunit downregulation in fish exposed to the contaminated sediments (especially $C_1$, most contaminated by metals, in both laboratory and *in situ* assays) comparatively to the reference condition likely resulted in an unbalancing of multiple protein regulation and recycling processes.

*4.3. Alterations to basal metabolism induced by toxicant stress*

Three energy production enzymes were found to be deregulated by exposure to contaminated

sediments, namely Eno1 and Eno3, which take part in the glycolytic pathway, and LDH, involved in anaerobic respiration. Increased regulation of glycolytic pathway enzymes has been found to occur in animals exposed to toxicants, e.g., in the kidney of rats exposed to bromates (Ahlborn et al., 2009) or in the liver of mice dwelling in metal-polluted sites (Montes-Nieto, et al., 2007). However, Biales et al. (2011) reported increased regulation of glycolytic enzymes in the brain of fathead minnows (*Pimephales promelas*) as a consequence of exposure to individual pesticides and a downregulation as a result of co-exposure. Other authors found that Cd may downregulate glycolytic enzymes in fish liver (*Micropterus salmoides*) with evidence for an inverse effect resulting from exposure to the PAH phenanthrene (Sanchez et al., 2009). It is likely that exposure to the contaminated sediments altered the respiratory metabolism, although with different outcomes resulting from the laboratory and field bioassays, presumably by differential bioavailability of contaminants. As before, it can be suspected that laboratory-exposed fish were more affected by co-exposure to metallic and organic contaminants, impairing the regulation of energy production enzymes, whereas field-tested soles revealed greater effects caused by organic substances which resulted in increased expression of enolase enzymes.

Laboratory exposure to $C_1$ and $C_2$ may have decreased energy production by downregulation of enolase, to which is added PGAM2 in $C_1$-tested fish, when compared to the reference test (Fig. 4.4.5). The resulting unbalance of the respiratory pathway can aid explaining why the anti-oxidant 1--cysPrx was upregulated in fish exposed to the contaminated sediments during the laboratory assays, since disruption of aerobic respiration can cause a net loss of available ATP in cells and an increase in ROS (see, e.g., Lemasters and Nieminen, 1997). In fact, 1-cysPrx was found allocated in the same cluster than respiration-related enzymes (Eno 1 and 3 plus PGAM2), depicting some degree of correlation in their expression, together with the glutamine synthetase and the lipid transporter ApoA1, both related to other baseline cellular processes such as aminoacid biosynthesis and lipid transport, respectively (Fig. 4.4.4). Only the allocation of PSB9 within this group remains unclear. Interestingly, PGAM2 displayed an almost opposite trend in regulation compared to the enzyme LDH, intervening in the pyruvate fermentation to lactate therefore recycling NADH back to NAD (Fig. 4.4.5), which may indicate that impairment of aerobic production of ATP increased the demand for anaerobically--produced energy.

Deregulation of enzymes of basal cell function (without a known direct function in response to toxic agents) has been described in several other works with fish liver or liver-derived cell lines. The function of such proteins ranges from energy production and aminoacid metabolism (e.g. Malécot et al., 2009; in a study with fish exposed to a microcystin toxin) to gluconeogenesis and cell cycle progression (Kling and Förlin, 2009; reported on fish exposed to brominated flame retardants). Concurrently, research employing transcriptomics provided similar results (e.g. Evrard et. al., 2010; on fish exposed to mixed herbicides). Downregulation of glycolytic enzymes and upregulation of key gluconeogenic enzymes could show a modulation of the carbohydrate/energy metabolism to produce sufficient reducing power for increased antioxidant needs in response to pollution (Montes-Nieto, et al., 2007). It can thus be inferred that deregulation of basal cellular functions is an important

consequence of xenobiotic-induced stress. Furthermore, it is highly plausible that impairment of baseline cell and tissue functions brings prejudice to the triggering of the necessary mechanism of response and defence against chemical injury. This constitutes the "disturbance" phase of the stress model discussed by other authors, during which organisms suffer a general imbalance that may lead to either a phase when appropriate responses are finally triggered or to biological failure if stress occurs too fast and/or is to strong to permit some form of adaptation (Steinberg et al., 2008). It is therefore possible that laboratory-tested soles, exposed to more bioavailable contaminants, suffered more pronouncedly the effects of metabolic imbalance and failed to resolve adequate responses to toxicity.

### *4.4. The potential role of oxidoreductase enzymes in response to chemical insult*

Oxidative stress has long been recognized as a keystone consequence of cellular metabolic imbalance caused, for instance by exposure to toxic agents. It constitutes a pivotal factor that surges directly (e.g. via CYP catabolism of organic compounds like PAHs) or indirectly (via a respiratory outburst triggered by stress and tissue injury and inflammation) that activates a wide range of cellular responses, from cell death (by necrosis or apoptosis) to cell proliferation or induction of DNA repair enzymes, among many other vital mechanisms of response and defence to chemical injury (refer to Roberts et al., 2010, for a review).

Besides energy production-related enzymes, the present results showed that three oxidoreductase enzymes (HSD, LDH and Aldh) were deregulated by exposure to the contaminated sediments, especially in laboratory-assayed fish, where, for the exception of LDH, the proteins 1--cysPrx, HSD, Aldh plus Eno 1 and 3 were deregulated in fish exposed to both $C_1$ and $C_2$ compared to R-tested animals. Among this subgroup, only 1-cysPrx was in fact upregulated when compared to the reference treatment. Regarding the *in situ* assays, only Aldh (in $C_2$-tested animals) was significantly upregulated compared to R-tested fish.

Aldehyde dehydrogenases are mitochondrial or cytosolic (as in the present study, since microsomal proteins were not surveyed) considered to take part in the detoxification of oxidant substances such as lipid peroxides (Zhang et al., 2010) and the ethanol metabolite acetaldehyde (Yoshida et al., 1989). Aldehyde dehydrogenase has been reported to be significantly downregulated in the crab *Carcinus maenas* collected from metal-polluted sites (Montes-Nieto, et al., 2010) as well as in the mussel *Mytilus galloprovincialis* (Nasci, et al., 2002), in this case due to the antagonistic effects of different contaminants. In contrast, this enzyme has also been found to have its activity increased as a result of exposure to environmental contaminants such as the TCDD (2,3,7,8-tetrachlorodibenzo-*p*--dioxin) dioxin and to the PAH methylcholanthrene (Nakanishi et al., 1979). Regarding HSDs (of which 17β HSDs are a just a family of a large group of known HSDs), although regarded as taking part in steroid hormone metabolism (and for such reason surveyed in the monitoring of endocrine disruptor compounds), they are suspected to have a broad-range of substrates, including xenobiotic by--products, due to their ability to act on carbonyl groups (Odermatt and Nashev, 2010). Albertsson et al.

(2007), for instance, found that a HSD enzyme (20β HSD) was upregulated in fish exposed to sewage effluents even in the absence of upregulation by the oestrogenic compound 17α ethinylestradiol, suggesting other compounds to be linked to deregulation of the enzyme.

In general, dehydrogenases, including HSD, LDH and Aldh (which are NAD/NADP dependent enzymes), have long been recognized important phase I detoxification enzymes (Oppermann and Maser, 2000). The Aldh enzyme was observed to be very significantly upregulated in field-exposed fish to sediment $C_2$ (most contaminated by organic compounds), which again may indicate that organic contaminants caused a more pronounced effected *in situ*. On the other hand, HSDs were most significantly downregulated by laboratory-exposure to the two most contaminated sediments, a deregulation that may have been a consequence of exposure to a more bioavailable mixture of contaminants and may have further subjected the fish to xenobiotics and their oxidant by--products.

### *4.5. Concluding remarks*

The regulated and identified proteins constitute fragments of complex cellular mechanisms potentially triggered by chemical stressors. Whether by 2DE limitations in spot detection of by failing to identify specific peptides by the MS/MS analyses (mostly due to the yet poor sequencing and annotation of teleost genomes and peptidomes), many key proteins involved in the above discussed processes could not be pin-pointed by the present study. Nevertheless, the present results show that a proteomic survey can detect proteins that take part in vital processes of the cell and are deregulated by contaminant action. These processes include basal metabolism, anti-oxidant defences and contaminant detoxification. In spite of their technical limitations, high throughput, “omic” approaches have proven value for both predictive and mechanistic toxicology, especially since they do not require a complete *a priori* knowledge of the cellular processes involved in the responses and defences to exposure, which is yet another great asset when surveying complicated contaminant matrices as sediments. For such reason, “omic” approaches, and proteomics in particular, are solid tools for ERA, especially in situations where initial screenings reveal mixtures of contaminants or other factors that may compromise classic biomarker approaches.

The most notorious difference between laboratory and *in situ*-exposed fish to contaminated sediments is the reduced induction of marked downregulation of most identified proteins in laboratory-exposed fish. It is highly probable that that increased bioavailability, combined with the presence of a mixture of different classes of contaminants, contributed the most for the accounted differences between laboratory and *in situ* exposed fish, which sustains the premise that laboratory assays can be more conservative. Conversely, caged fish exposed to contaminated sediments appear to respond more pronouncedly to contamination by organic substances, with fish exposed to sediment $C_2$ (the most contaminated by PAHs and organochlorines) presenting the most dissimilar proteins response pattern comparatively to fish exposed to the reference (clean) sediment, which likely

indicates a higher pressure from chemical stressors. It must also be recognized that, more importantly than finding specific up- or downregulated protein responses, especially when dealing with complex mixtures of xenobiotics, deregulation itself can be the best indicator of stress. Even though more classic protein biomarkers such as metallothionein or CYP enzymes are often based on the premise that exposure causes induction, it is clear that chemical stress may cause downregulation of many protein responses, for instance as a result of general metabolic impairment, at least during the first stages of chronic exposure. From the current findings it may also be inferred that the choice of bioassay type is crucial. If laboratory assays may provide a more complete scenario on the sediments' full toxicological impact, *in situ* assays, on their turn, should permit a more realistic approach determination of the true risks of contamination towards the biota.

## Acknowledgements

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors would also like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA); J. Raimundo, V. Branco, R. Cesário and P. Pousão (IPIMAR-INRB) and Carlos A. Fuentes Almagro and Ricardo Fernández Cisnal (Universidad de Córdoba), for their important support.

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# Chapter 5. Novel approaches on the mechanisms of toxicity

## 5.1. Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]pyrene[†]


### Abstract

The livers of soles (*Solea senegalensis*) injected with subacute doses of cadmium (Cd), benzo[a]pyrene (B[a]P), or their combination, were screened for alterations to cytosolic protein expression patterns, complemented by cytological and histological analyses. Cadmium and B[a]P, but not combined, induced hepatocyte apoptosis and Kupfer cell hyperplasia. Proteomics, however, suggested that apoptosis was triggered through distinct pathways. Cadmium and B[a]P caused upregulation of different anti-oxidative enzymes (peroxiredoxin and glutathione peroxidase, respectively) although co-exposure impaired induction. Similarly, apoptosis was inhibited by co--exposure, to which may have contributed a synergistic upregulation of tissue metalloproteinase inhibitor, β-actin and a lipid transport protein. The regulation factors of nine out of eleven identified proteins of different types revealed antagonistic or synergistic effects between Cd and B[a]P at the prospected doses after 24 h of exposure. The results indicate that co-exposure to Cd and B[a]P may enhance toxicity by impairing specific responses and not through cumulative damage.



### Keywords

Metal; Polycyclic aromatic hydrocarbon; Proteomics; Apoptosis; Hepatic parenchyma


### 1. Introduction

The mechanisms underlying cellular detoxification and elimination of xenobiotics are complex and are known to depend on multiple factors such as contaminant class, doses, biological species, affected tissue and cell types, and co-exposure to other contaminants. The complexity of these mechanisms in whole-tissue and organs has been an important constraint to many fields of xenobiotic research, such as environmental toxicology and human occupational health, especially when multiple contaminants are involved. Most toxicological studies that focused on contaminant interactions aimed at the effects on common biomarkers, or potential biomarkers, such as metallothionein (MT), and

[†] Costa et al. (2010). *Environ. Pollut.* **158**(10), 3338-3346 (doi:10.1016/j.envpol.2010.07.030).

cytochrome P4501A (CYP1A) induction, activity of antioxidant enzymes, etc. (e.g. Sandvik et al., 1997; Hurk et al., 1998; Orbea et al., 2002; Marigómez et al., 2005; Costa et al., 2009a; Rosijadi et al., 2009). Such studies revealed the existence of antagonistic (opposite effect) and synergistic (a combination that produces an outcome that is higher than the sum of each isolated xenobiotic) traits when an organism, organ or cell culture is exposed to multiple contaminants.

Cadmium (Cd) and benzo[a]pyrene (B[a]P) are common contaminants in industrialized areas, especially affecting coastal water bodies, where organic enriched sediments or suspended particles function as a trap for metals such as Cd and hydrophobic organic xenobiotics like polycyclic aromatic hydrocarbons (PAHs), among which B[a]P is included. Airborne particle-bound Cd and B[a]P (e.g. fumes and ashes) are also of great concern to environmental and, especially, human health (Harris et al., 1985; Viaene et al., 2000). For such reasons, many studies have been carried out to evaluate Cd and B[a]P toxicity in aquatic and terrestrial environments (see Friesen et al., 2008 and Nordberg, 2009 for a review). Both xenobiotics have also been widely employed as model toxicants in *in vivo* and *in vitro* studies. Nevertheless, interaction studies are scarce and even scarcer with respect to whole--tissue/organ *in vivo* effects.

Toxicity of PAHs, including B[a]P, is mostly linked to the production of activated PAH forms such as PAH quinones and the highly genotoxic diol epoxides by the CYP1A monooxygenase complex with subsequent release of reactive oxygen species, ROS (Conney, 1982; Flowers-Geary et al., 1996). Consequently, the majority of metal + PAH interactions have focused on oxidative stress and mutagenicity. Cadmium and other metals have been found to reduce the activity and induction of CYP1A in human HepG2 cells exposed to B[a]P even in presence of non-cytotoxic concentrations of the metals (Vakharia et al., 2001). Cadmium is known to be toxic at low levels of exposure but the exact mechanisms of toxicity are not yet fully understood. It is thought that Cd may displace iron and zinc from metallothioneins and other proteins such as copper-zinc superoxide dismutase (CuZn SOD) and from zinc-finger class proteins (Bauer et al., 1980; Asmuß et al., 2000; López-Barea and Gómez--Ariza, 2006). Synergistic and antagonistic effects have been found on lethality induced by intraperitoneally injected Cd and B[a]P in fish, which was speculated to be caused by Cd-induced reduction of CYP1A activity and metallothionein inactivation by B[a]P (Hurk et al., 1998). Although Cd is regarded as a genotoxicant on its own, it has been found to enhance DNA damage induced by a B[a]P epoxides metabolite by impairment of DNA repair in human HeLa cell extracts in a dose--dependent pattern (Mukherjee et al., 2004). Conversely, no interaction effects were found regarding the occurrence of clastogenic nuclei of mouse bone marrow cells (Lewińska et al., 2007). However, the effects of co-exposure to metals and PAHs on cell and tissue structure and biochemistry (including detoxification and regeneration) still remain largely unknown.

In an attempt to study Cd and B[a]P interactions on a species realistically subjected to potential metal and PAH exposures, we have chosen the Senegalese sole *Solea senegalensis* Kaup, 1858 (Teleostei: Soleidae) as a test species. This species is a common estuarine flatfish in southeastern Europe that has been employed in a growing number of environmental monitoring and baseline

toxicological studies (e.g. Jiménez-Tenorio et al., 2007; Costa et al., 2008; 2009a, 2009b). The combined effects of metallic and organic contaminants (especially PAHs) in biomarkers of exposure and effect in *S. senegalensis* have already been reported to yield unexpected results in fish exposed to estuarine sediments, revealing, at some extent, interaction effects between the two classes of contaminants (Costa et al., 2008, 2009a, 2009b). For such reason, the present work intends to explore the mechanisms of metal × PAH interaction, employing Cd and B[a]P as model xenobiotics. The present study aims at the effects and responses of Cd and B[a]P in whole-liver tissue combining proteomics, histology and cytology as screening tools. It is intended to contribute to the understanding of the immediate mechanisms of Cd and B[a]P co-exposure and their consequences in the hepatic parenchyma of a species that is exposed in its natural habitat to mixed classes of contaminants, including metals and PAHs.

## 2. Materials and methods

### *2.1. Experimental procedure*

Twenty-four randomly selected laboratory-hatched *S. senegalensis* juveniles (52.7 ± 1.3 mm standard length, 2.2 ± 0.4 g total wet weight) from the same cohort were divided by four experimental treatments: control, Cd, Cd + B[a]P and B[a]P. Tests were performed in duplicate, with three individuals per replicate. All tested fish were intraperitoneally injected with the same carrier solution containing 5 µL Milli-Q grade ultrapure water + 10 µL ≈ 100% dimethyl sulfoxide (DMSO) + 85 µL sterilized 50 mM PBS (pH 7.4, with 0.7% sodium chloride). The injected Cd dosage was obtained by preparing the carrier solution with 5 µL of 1 $\mu g.\mu L^{-1}$ cadmium chloride solution in Milli-Q water diluted from a standard $CdCl_2$ Tritisol solution (Merck). The B[a]P dosage was achieved by preparing the carrier with 10 µL of 10 $nmol.\mu L^{-1}$ B[a]P (from Sigma) diluted in ≈ 100% DMSO. Table 5.1.1 summarizes the procedure. Control individuals were injected with the carrier solution prepared without any of the test xenobiotics. Real injected dosages were 24.0 ± 4.5 nmol Cd $g^{-1}$ fish wet weight (2.7 ± 0.5 µg Cd $g^{-1}$) and 5.1 ± 1.0 nmol B[a]P $g^{-1}$ fish wet weight (1.3 ± 0.3 µg B[a]P $g^{-1}$). Doses were determined to ensure subacute administration of contaminants, according to pre-existing data (Hurk et al., 1998). After injection, animals were incubated for 24 h in 15 L polyvinyl tanks with 9 L of filtered seawater, with continuous aeration, at constant conditions (temperature = 18 ± 1 ºC, salinity = 33 ± 1, pH 8.0, total ammonia ≈ 0 $mg.L^{-1}$). Animals were collected after the incubation period, euthanized by decapitation and liver samples collected for subsequent analyses.

**Table 5.1.1.** Summary of the experimental procedure.

| **Experimental treatment** | **Injected Cd (µg)** | **Injected B[a]P (nmol)** | ***N*** |
|---|---|---|---|
| Control | - | - | 6 ($2 \times n = 3$) |
| Cd | 5 | - | 6 ($2 \times n = 3$) |
| Cd + B[a]P | 5 | 10 | 6 ($2 \times n = 3$) |
| B[a]P | - | 10 | 6 ($2 \times n = 3$) |

*N* - total number of individuals per experimental treatment; *n* - number of individuals per replicate

*2.2. Proteomic analysis*

The differential expression of cytosolic proteins was assessed by two-dimensional electrophoresis (2DE), through a protocol adapted from previous research (Romero-Ruiz et al., 2006; Montes-Nieto et al., 2007). For each experimental condition ≈ 100 mg of liver samples were pooled and homogenized in liquid nitrogen prior to extraction in 20 mM Tris-HCl buffer (pH 7.6) with 0.5 M sucrose and 0.15 M KCl, complemented with 20 mM dithiothreitol (DTT) as a reducing agent; 1 mM phenylmethylsulfonyl fluoride (PMSF), 6 µM leupeptin and 100 $\mu L.mL^{-1}$ of the Protease Inhibitor Cocktail (Sigma) as proteolytic activity inhibitors. After centrifuging for 1 min (to remove the lipid supernatant) + 10 min (4 ºC at 14,000 × g), the samples were treated with 500 $U.mL^{-1}$ benzonase endonuclease for 30 min at room temperature, followed by centrifuging at 102,000 × g for 1 h (at 4 ºC) to precipitate remaining non-peptide material. The samples' total protein was quantified according to Bradford (1976) in order to load each IPG strip (18 cm, pH 4-7; from GE Healthcare) with 100 µg of protein. From this point onwards, the procedure was performed in triplicate per each experimental condition (3 IPG strips + 3 gels per condition). The IPG strips were incubated for 30 min at room temperature with pH 4-7 IPG buffer (GE Healthcare) including 7 M urea, 2% w/v CHAPS (a non--ionic detergent), 20 mM DTT and ≈ 1% w/v bromphenol blue. After passive rehydration (6 h, 20 ºC), strips were subjected to isoelectric focusing (IEF) on a Protean IEF apparatus (Bio-Rad). Separation by protein subunit molecular weight (MW) was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 18 × 18 cm 12.5 % acrylamide/bis-acrylamide gels after equilibration of IPG strips in 1.5 M Tris-HCl buffer (pH 8.8) with 6 M urea and 2% SDS (as denaturing agents); 65 mM DTT, 3 % glycerol and ≈ 1% w/v bromphenol blue, which was followed by a treatment with 25 mM iodacetamide (IAA) to block sulphydryl groups. Electrophoresis was run at constant wattage (10 W per gel) in a DodecaCell Plus system from Bio-Rad. The SigmaMarker wide-range protein ladder (Sigma-Aldrich) was used as MW standard. Gels were afterwards stained with Sypro Ruby fluorescent dye (Bio-Rad) and imaged with a Bio-Rad FXImager laser scanner. Gel analyses and protein regulation factors were achieved using the software ProteomWeaver (Bio-Rad). Spots were selected

for protein identification according to the following criteria: a significant difference in spot intensity ($\alpha$ = 0.05, Mann-Whitney *U* test) of at least one experimental condition comparatively to control gels; a minimum of 150% or 50% up- or downregulation compared to control gels, respectively, consistent within the three gels obtained per experimental condition, to ensure good contrast between regulation factors (e.g. Montes-Nieto et al., 2007). Excised spots were treated with DTT and IAA prior to digestion with porcine trypsin. The products were sequenced by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) using a Voyager De-PRO apparatus (Applied Biosystems) to evaluate digestion quality and eliminate trypsin self-proteolysis noise, followed by *de novo* sequencing through capillary liquid chromatography electrospray ionization ion trap tandem mass spectrometry (capLC-ESI-ITMS/MS) with the LTQ system from ThermoFisher Scientific. Peptide search was performed using the software Protein-Protein Blast 2.2.20 (Altschul et al., 1997) to contrast results to chordate, actinopterygian and pleuronectiform peptide sequences from the NCBI All-Non Redundant (nr) Protein Sequence database and validated by the criteria of highest score, lowest *e*-value, number of matched peptides and representativity in the prospected taxa sets.

*2.3. Microscopy analyses*

Liver samples were fixed in Bouin-Hollande's solution for 36 h, dehydrated in a progressive series of ethanol, embedded in paraffin and sliced to 1-3 µm thick sections (Martoja and Martoja, 1967). Sections where either stained with haematoxylin and eosin (H&E) for histological evaluation or with the acridine orange (AO) nucleic acid-binding fluorochrome for determination of apoptosis. Additional liver samples were fixed with a glutaraldehyde and paraformaldehyde mixture in 0.1M cacodilate buffer (pH 7.2) with 2.5 % w/v NaCl and 6 % sucrose and postfixed with osmium tetroxide before dehydration in a progressive series of ethanol and embedding in LR White resin. Sections (200--400 nm thick) where stained with toluidine blue for structural detailing, sudan black B for lipids (Bronner, 1975) and Coomassie brilliant blue (CBB) R250 for peptides (Fisher, 1968). The proportion of apoptotic and normal hepatocytes was determined on AO-stained slides (Okada et al., 2002), observed with UV epifluorescence at a × 1,000 magnification. At least 1,000 cells per individual were counted from eight to twelve sections per slide. Apoptotic cells were scored following morphologic criteria (Häcker, 2000).

All analyses were performed with a DMLB model microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps. The optical path was equipped with an I3 filter to detect AO fluorescence. All equipment was supplied by Leica Microsystems. Image analyses and processing was done with the software ImageJ (Wayne Rasband National Institutes of Health, USA).

*2.4. Statistical analyses*

Altered protein regulation factors relatively to controls were analyzed by cluster analysis based on the 1-Pearson *r* statistic in order to determine the degree of correlation between the expressions of identified proteins. The differences between the percentages of apoptotic cells in hepatic tissue were assessed through the non-parametric Mann-Whitney *U* test. The statistical significance level was set at $\alpha = 0.05$ for all analyses. Statistics were computed using the software Statistica (Statsoft Inc.).

## 3. Results

*3.1. Proteomic analysis*

The analysis of changes in protein regulation patterns by 2DE yielded twenty-four protein spots that met the assumptions abovementioned and were thus selected for protein identification. Eleven of these twenty-four spots provided a positive identification after contrasting to the NCBI nrProtein database the MS/MS spectra obtained by *de novo* sequencing of several peptides from each protein that had been selected as suitable precursors in the previous MALDI-TOF analysis. Peptides potentially resulting from keratin contamination were eliminated from subsequent analyses. Due to the small number of *S. senegalensis* sequenced peptides deposited in public access databases, only β-actin and trypsin/trypsinogen were directly matched to the species. The best-matched protein was cathepsin L (CatL) with a 90.1 score and a $4.0 \times 10^{-19}$ *e*-value (Table 5.1.2).

Two proteins, 1-cys peroxiredoxin (1-cysPrx) and CatL were observed to be significantly upregulated (overexpressed relatively to control fish) as a consequence of Cd treatment. Cell division cycle 48 (CDC48) was observed to be downregulated (underexpressed) as a consequence of the B[a]P treatment. Five proteins were differentially expressed in fish treated with Cd + B[a]P. The remaining proteins revealed expression patterns significantly different from controls by two of the three different xenobiotic treatments, although in some cases with clear opposite responses such as in histone H4, upregulated in Cd and downregulated in B[a]P treatments. Fig. 5.1.1 summarizes the different regulation patterns observed comparatively to controls.

**Table 5.1.2.** Protein identification summary after *de novo* sequencing using ESI-ITMS/MS and peptide sequence database search with Protein-Protein Blast plus relative regulation factors over control (± standard deviation) for each identified protein.

| | | | | | | | Regulation factors over control | | |
|---|---|---|---|---|---|---|---|---|---|
| **Protein ID** | **Abbreviation** | **UniProt Accession** | **Taxa database†** | **Score** | ***e*-value** | **N° matched peptides** | **Cd** | **Cd + B[a]P** | **B[a]P** |
| 1-cys Peroxiredoxin | 1-cysPrx | B5X838 | 1,2,3 | 36.7 | $4.0 \times 10^{-5}$ | 2 | 0.64 ± 0.44 | 0.30 ± 0.44 | -0.21 ± 0.16 |
| Apolipoprotein A-IV3 | ApoA-IV3 | Q5KSU2 | 2,3 | 29.1 | $8.7 \times 10^{-1}$ | 6 | -0.20 ± 0.27 | 0.54 ± 0.23 | -0.25 ± 0.18 |
| Beta-actin | β-actin | Q1HHC7 | 1,2,3 | 37.1 | $3.0 \times 10^{-5}$ | 5 | -0.51 ± 0.10 | 0.91 ± 0.50 | 0.46 ± 0.28 |
| Cathepsin L | CatL | P79722 | 1,2,3 | 90.1 | $4.0 \times 10^{-19}$ | 4 | 0.61 ± 0.07 | 0.40 ± 0.06 | 0.13 ± 0.07 |
| Cell division cycle 48 | CDC48 | A5JP17 | 1 | 30.3 | $4.0 \times 10^{-3}$ | 2 | -0.39 ± 0.10 | -0.44 ± 0.11 | -0.52 ± 0.04 |
| Glutathione peroxidase | Gpx | Q802G1 | 2,3 | 28.6 | $1.2 \times 10^{0}$ | 2 | 0.30 ± 0.17 | 0.76 ± 0.10 | 1.42 ± 0.32 |
| Histone H4 | H4 | H4 | 2,3 | 35.0 | $1.5 \times 10^{-2}$ | 2 | 1.93 ± 0.98 | 0.08 ± 0.32 | -0.50 ± 0.05 |
| Metallothionein I | MT1 | MT1 | 1,2,3 | 38.8 | $8.0 \times 10^{-3}$ | 3 | -0.38 ± 0.14 | -0.51 ± 0.04 | -0.50 ± 0.07 |
| Phosphatidylethanolamine-binding protein | PEBP | B5DGG2 | 2,3 | 37.5 | $2.0 \times 10^{-2}$ | 3 | -0.48 ± 0.03 | -0.54 ± 0.06 | 0.35 ± 0.14 |
| Tissue metalloproteinase inhibitor 2 | TIMP2 | B5XCZ1 | 1,2,3 | 46.9 | $4.0 \times 10^{-6}$ | 1 | 0.13 ± 0.21 | 0.52 ± 0.48 | 0.40 ± 0.54 |
| Trypsin | Trypsin | Q5XUG5 | 1,2,3 | 24.8 | $1.7 \times 10^{-1}$ | 5 | 0.46 ± 0.06 | 0.68 ± 0.17 | 0.12 ± 0.23 |

†nrNCBI database taxa from which peptides were matched: 1-Order Pleuronectiformes; 2-Class Actinopterygii; 3-Phylum Chordata

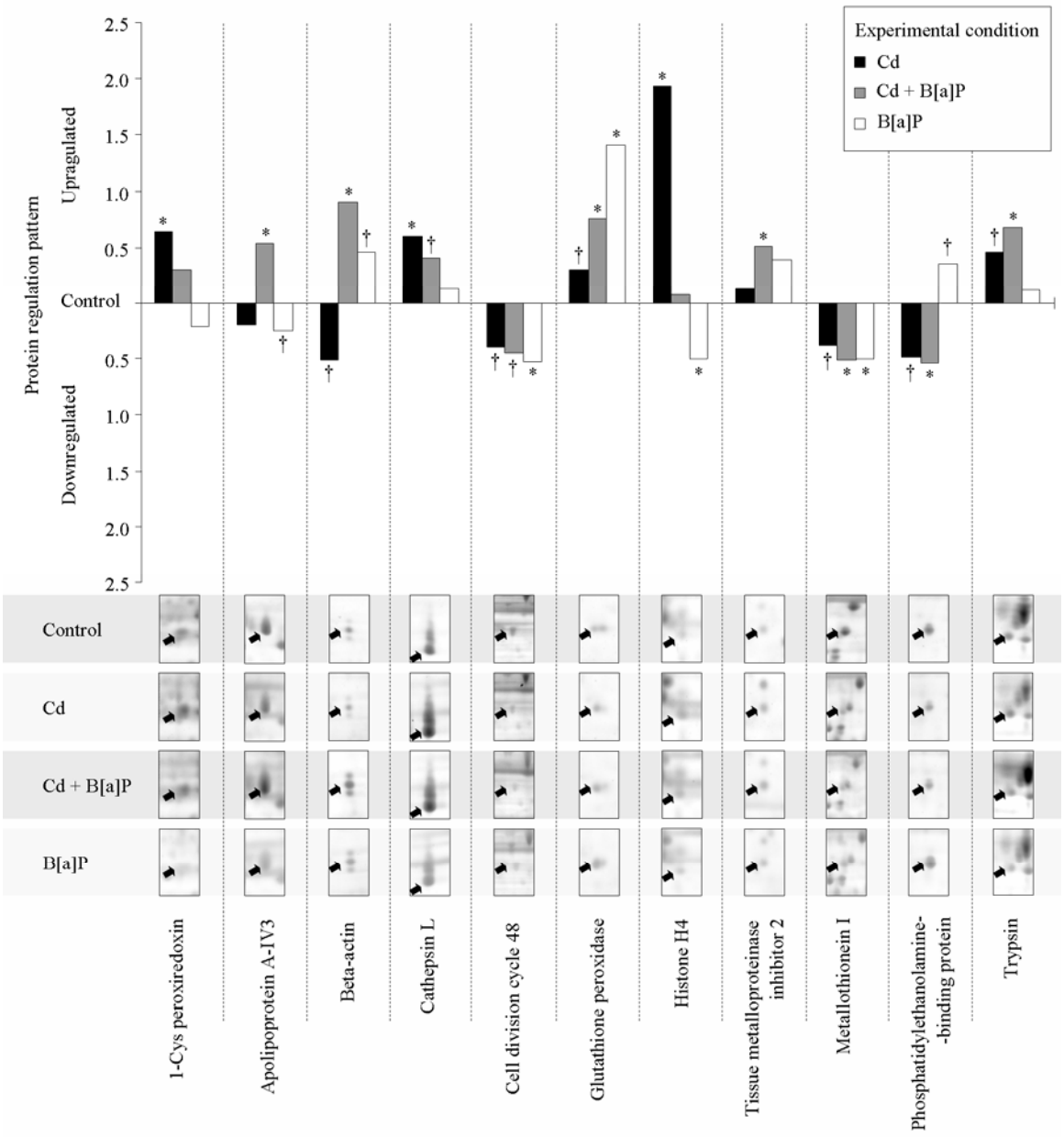


**Fig. 5.1.1.** Average protein regulation factors (expressed by an arbitrary unit relatively to control) and respective 2DE gel photographs for each spot (arrows). [*] significant differences of spot intensities from controls gels (Mann-Whitney *U*, $p < 0.05$) and regulation factor up or down 150% and 50%, respectively, relatively to control gels. [†] significant differences of spot intensities from control gels (Mann-Whitney *U*, $p < 0.05$) but regulation factors narrower than the imposed thresholds of control ± 50% protein expression.

*3.2. Liver histology.*

Fish injected with Cd, B[a]P or both contaminants exhibited very considerable alterations in hepatic parenchyma in comparison to controls (Fig. 5.1.2). H&E-stained hepatocytes from control fish were observed to be polyedric in shape and presented a translucent, virtually unstained, cytoplasm with few inclusions and a spherical nucleus with a centric nucleolus. The parenchyma contained many

sinusoids anastomosing from branches of the hepatic portal vein. Although the basic hepatic architecture did not appear to be significantly altered in fish treated with Cd, B[a]P or Cd + B[a]P, the livers of individuals injected with the isolated xenobiotics presented a higher density of cells caused especially by a profusion of hepatic-specific macrophages (Kupfer cells). This increment in cell density caused hepatocytes to compress, which, combined with an increase in intracellular inclusions (including lipid vacuoles), provided an overall more intense H&E staining of the tissue, making it difficult to detect cell boundaries and identify cell types (Fig. 5.1.2A). Oppositely, the hepatic parenchyma of individuals treated with Cd + B[a]P presented a less significant intrusion of defence cells. Hepatocytes of these fish, however, contained many densely-stained intracellular structures although empty-like lipid vacuoles where rare or absent. Necrosis was confined to small, sparse, foci in livers of fish treated with Cd, B[a]P and Cd + B[a]P but rarely observed in controls. It is not clear if Cd + B[a]P livers presented more necrotic foci or if these were more conspicuous due to less dense cell packing. Late stage inflammatory responses, such as blood vessel profusion and dilation and retention of erythrocytes within the parenchyma, were not observed in any treatment. Apoptotic hepatocytes, especially at a later stage of PCD (programmed cell death), were easily distinguishable in AO-stained sections (Fig. 5.1.2B). Apoptotic cells were observed in the livers of individuals subjected to all treatments but with a greater incidence in fish injected the isolated toxicants. Sudan staining of semi-thin sections confirmed the presence of large lipid vacuoles (lipidosis) in hepatocytes of Cd- and B[a]P-treated fish (Fig. 5.1.2C). Lipid droplets in controls were limited to sparse microvesicles and virtually absent from livers treated with Cd + B[a]P.

*3.3. Cytological observations and hepatic cell apoptosis.*

Kupfer cell intrusions are identifiable by stronger intraplasmatic retention of haematoxylin and AO pigments than hepatocytes due to a high concentration of phagosomes and lysosomes (Fig. 5.1.3A, B). Besides its affinity to nucleic acids, acridine orange is a weak base known to accumulate in acidic intracellular compartments such as proteolitic lysosomes (Völkl et al., 1993). These macrophages, normally associated to sinusoids, were observed to proliferate (hyperplasia) and intrude into hepatic parenchyma from adjacent blood vessels in Cd- and B[a]P-treated individuals. Kupfer cell hyperplasia was not observed in controls and fish treated with Cd + B[a]P. Apoptotic cells (Fig. 5.1.3C-E) were mostly observed, alone or in clusters, near blood vessels, usually compressed between normal hepatocytes and Kupfer cells. Cytological analyses identified intracellular inclusions in macrophages and hepatocytes probably as lysosomes and phagosomes (deeply stained by the Coomassie blue stain for peptides) containing the remains of apoptotic, necrotic or authophagic cells (Fig. 5.1.3F). Oval (stem) cells could often be found intruding the hepatic parenchyma from the portal vein branches, more significantly in Cd and B[a]P treatments. Necrotic and autophagic cells could be observed in livers from all treatments but were found difficult to pinpoint individually and, occasionally, even to discriminate. For such reason they could not be objectively quantified. The counting of hepatocytes

undergoing apoptosis on AO-stained slides revealed that treatments with Cd and B[a]P significantly increased the percentage of apoptotic hepatocytes relatively to control (Mann-Whitney *U*, $p < 0.05$) but not in the combined Cd + B[a]P treatment (Fig. 5.1.4). However, no significant differences were found between Cd and B[a]P treatments (Mann-Whitney *U*, $p > 0.05$).

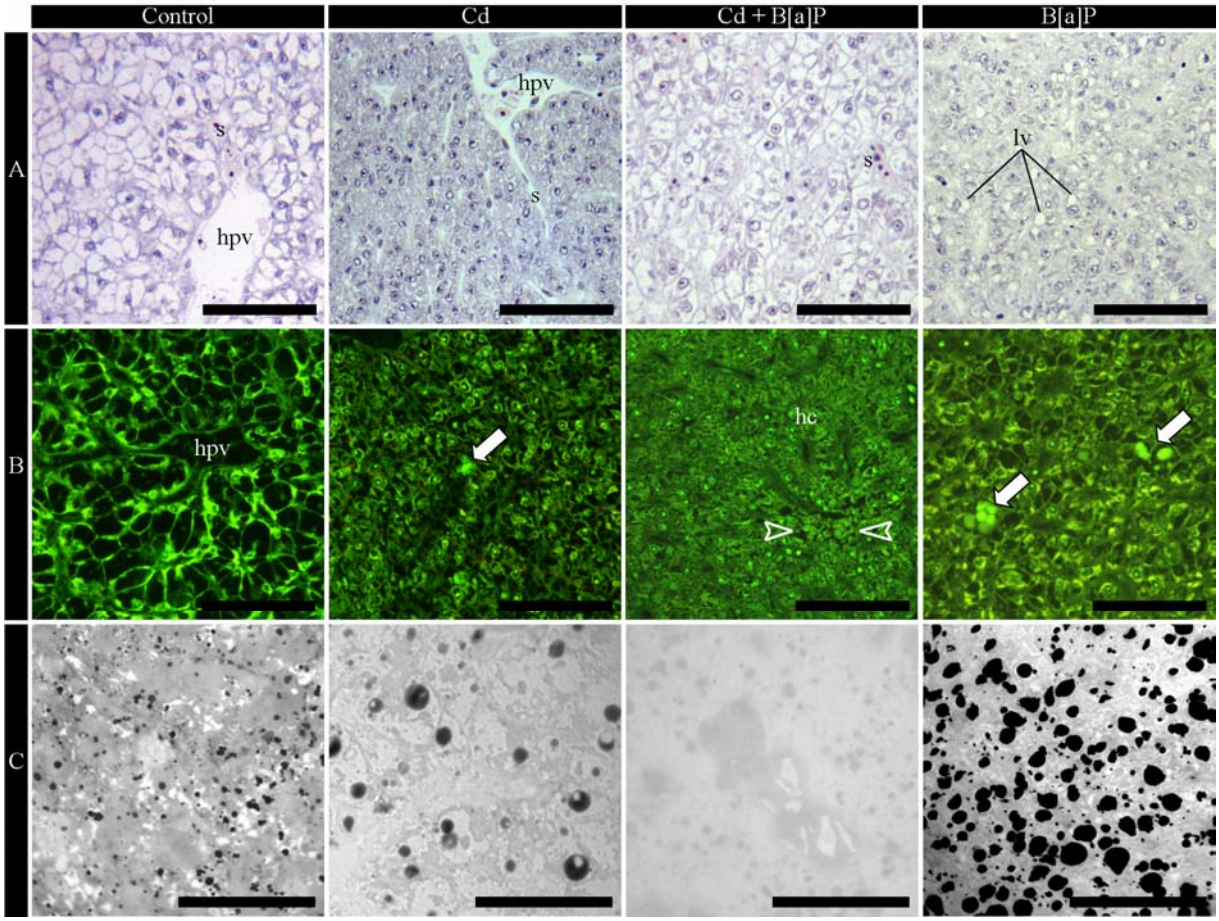


**Fig. 5.1.2.** Overview of hepatic parenchyma of tested individuals. hc) hepatic cord exhibiting the typical rosette--like structure of hepatocytes lining a transversally sectioned sinusoid; hpv) hepatic portal vein branch; **lv**, lipidic vacuoles; **s**, sinusoid. Scale bar: 25 µm. (A) Hepatic tissue stained with H&E. Cells of Cd- and B[a]P-injected individuals appear more densely packed than those of controls and Cd+B[a]P due to vacuolation and infiltration of defence cells in parenchyma. Hepatocytes of fish injected with both xenobiotics exhibit many intraplasmatic inclusions, likely to be phagosomes and lysosomes. Localized foci of necrotic cells were also found no be more frequent in fish subjected to this experimental condition. (B) Apoptotic cells (arrows) were found to be distributed without any evident pattern, isolated or in small clusters, typically compressed against blood vessels. Although present in fish subjected to any of the treatments, apoptotic cells were more frequent in individuals injected with Cd or B[a]P alone. AO stain, viewed with epifluorescent UV light. (C) Sudan black B stain of semi-thin sections. Lipid droplets (black inclusions) could be found in hepatocytes of control and Cd- or B[a]P--injected individuals but were virtually absent in liver cells of fish injected with both substances. However, while in hepatocytes from controls lipid bodies were present as microvesicles, large vacuoles were formed in fish injected with Cd or B[a]P, indicating severe hepatic lipidosis.

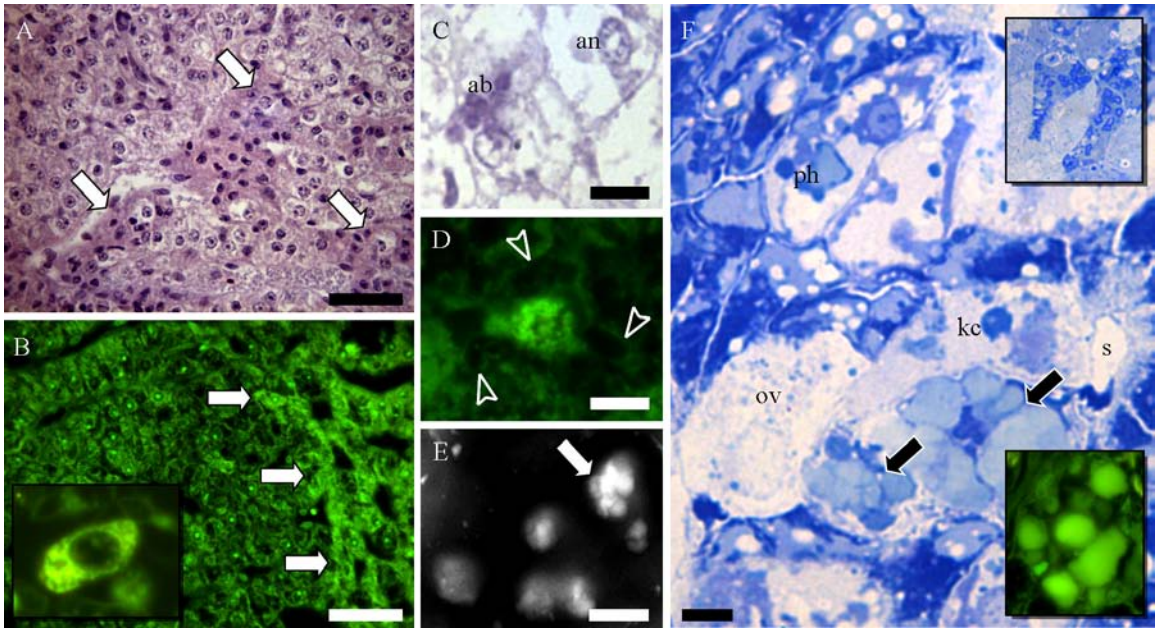


**Fig. 5.1.3.** Discrimination of hepatic cell types and anomalies. (A) Kupfer cell intrusion in the liver of a B[a]P--injected individual (arrows). H&E stain. Scale bar: 25 µm. (B) Same as previous, stained with AO and viewed under epifluorescent UV light. Kupfer cell hyperplasia (arrows) is clearly discriminated from adjacent tissue due to its strong fluorescence. Side panel: a Kupfer cell on a blood vessel. The cytoplasm of these cells exhibits strong fluorescence, likely due to a high concentration of acidic lysosomes. Batches of AO-stained heterochromatin are visible lining the nuclear envelope. Scale bar: 25 µm. (C) Apoptotic bodies compressed between hepatocytes (ab). An early-stage apoptotic nuclei (an) can be observed in an adjacent cell, showing chromatin condensation and compression against the nuclear envelope instead of the typical concentric nucleolus. H&E stain. Scale bar: 5 µm. (D) An intermediate stage of hepatocyte apoptosis viewed under epifluorescent UV light after AO staining. Chromatin fibres hypercondensate and begin to compress against the inner facing of the nuclear envelope, producing a strong fluorescent signal. The plasmatic membrane begins to bleb (arrowheads) as the cell shrinks in volume. Scale bar: 5 µm. (E) Three-dimensional rendering of a cluster of apoptotic cells (AO-stain and UV epifluorescence, obtained from six planes with a depth of approximately 0.5 µm). The formation of the highly-fluorescent apoptotic bodies is clear on the highlighted cell (arrow). Scale bar: 5 µm. (F) A semi-thin section of the liver of a Cd-injected individual stained with toluidine blue. Arrows highlight two apoptotic cells at an advanced stage. The nearly-detaching apoptotic bodies present a heterogeneous colouration due to the varying contents of each apoptotic pouch. A Kupfer cell (kc) is seen infiltrating the tissue from a nearby sinusoid (s), likely preparing to phagocytize the forming apoptotic bodies. Phagosomes containing indiscriminate material could often be found inside normal hepatocytes (ph). Side panel, upper right: two Kupfer cells penetrating the hepatic parenchyma exhibiting many protein-rich phagosomes (CBB stain). Side panel, lower right: overview of an apoptotic cell cluster similar to the one shown on the main figure (AO stain under UV epifluorescence). Scale bar: 5 µm.

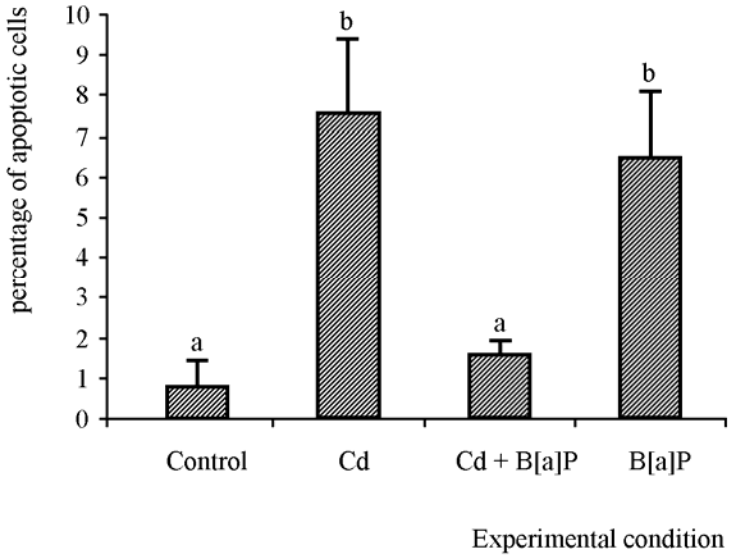


**Fig. 5.1.4.** Average percentage of apoptotic cells per experimental condition. Error bars represent 95% confidence intervals. Different letters mean significant differences (Mann-Whitney *U* test, $p < 0.05$).

## 4. Discussion

The present findings revealed an impairment in the induction of hepatocyte apoptosis and tissue regeneration as a consequence of a simultaneous *in vivo* short-term treatment with the low doses of Cd and B[a]P, accompanied by changes in protein expression patterns. Apoptosis is a pathway for PCD regarded as a mechanism to control the elimination of damaged cells avoiding dissemination of potentially harmful degradation products and excessive inflammatory response (Fedeel and Orrenius, 2005; Häcker, 2000). In addition, the observation of Kupfer and oval (stem) cells in fish treated with the isolated contaminants indicates that the liver rapidly triggered recovery mechanisms. Kupfer cells and adjacent hepatocytes are responsible for the removal of apoptotic bodies and other cellular debris while oval cells infiltrate from periportal areas to replace the regressing parenchyma. The combination of Cd and B[a]P at the given doses impaired the regeneration process beginning with the blocking of PCD. Oval cells, on the other hand, are known to intrude into the hepatic tissue when the normal regenerative process is insufficient to respond to mechanical or chemical insult (Oh et al., 2002). Previous research by Arai and co-workers (2004), for instance, showed that a wide range of genes are rapidly overexpressed in face of injury and subsequent oval cell proliferation, from energy balance--related genes to growth factors among which were included, for instance, apolipoprotein- and histone-related genes. However, the mechanisms of hepatic regeneration are yet poorly understood.

Although Cd and B[a]P treatments resulted in many similar alterations in hepatic tissue,

namely apoptosis, lipid vacuolation and Kupfer and oval cell intrusions, the proteomic analysis indicates that different biochemical pathways of response occurred. Most metal and PAH interaction studies focused on the effects on known biomarkers, leaving questions regarding the specific biochemistry of contaminant co-exposure yet to be answered. Marigómez et al. (2005), for instance, on the course of a single-concentration waterborne exposure experiment (500 $\mu g.L^{-1}$ B[a]P plus 80 $\mu g.L^{-1}$ Cd), found that Cd could inhibit lysosomal changes triggered by B[a]P in mussels. Sandvik et al. (1997) observed similar results in CYP1A induction and a limitation of Cd-induced metallothionein upregulation by B[a]P in the liver of flounder intraperitoneally injected with single-doses of the metal and the PAH similar to those employed in the same study (1 $\mu g.g^{-1}$ and 2.5 $\mu g.g^{-1}$, respectively). These results are in accordance with the findings on hepatic MT and CYP1A responses in *S. senegalensis* exposed sediments contaminated by mixtures of organic and metallic contaminants (Costa et al., 2009a). Interestingly, other authors found that co-exposure to B[a]P and Cd potentiated MT expression in the intestine of fish fed with contaminated pellets (Roesijadi et al., 2009), which may indicate that these interaction effects may depend on organ-specific xenobiotic uptake and elimination processes. Bioaccumulation, on the other hand, has been argued to be unaffected by co-exposure to metals and PAHs (Marigómez et al., 2005). The present findings confirm that the biochemistry of metal-PAH interactions in a vertebrate liver are complex and may involve a broad-range of protein responses with multiple consequences at the histological and cytological levels.

Correlation analysis between protein expression factors revealed possible links between expression patterns (Fig. 5.1.5). Whereas CDC48 and MT1 changes in regulation patterns appeared uncorrelated to the remaining proteins', the other proteins (excluding PEBP) formed two distinct clusters. The first cluster included H4, 1-cysPrx, CatL and Trypsin, whose overexpression may have been especially linked to Cd. The second cluster comprised TIMP2, β-actin, GPx and ApoA-IV-3 whose overexpression can be linked to B[a]P or to the interaction between B[a]P and Cd. CDC48 pertains to the AAA ATPase family (ATPases associated with diverse cellular activities), related to energy-dependent unfolding, disassembly and degradation of protein complexes, membrane fusion, microtubule severing, peroxisome biogenesis, signal transduction and the regulation of gene expression. They form a molecular motor that couples ATP hydrolysis to changes in conformational states of a target substrate, either translocating or remodelling it. Recent studies showed that cellular depletion of CDC48 affects continuance of cellular cycle by impairing DNA synthesis (Mouysset et al., 2008). Although DNA replication and induction of cell division by mitogens counterbalance apoptosis, the more specific role of CDC48 in the process remains unclear. It is likely that downregulation of CDC48 reflects a general failure of hepatic cell metabolism. Similarly, some evidence exists that MTs might prevent Cd-driven apoptosis (Kondo et al., 1997). Our findings, however, reveal an uncertain relationship between MT isoform 1 and alterations to liver histology even though B[a]P, alone or combined with Cd (a strong MT inducer), contributed to a more pronounced MT downregulation.

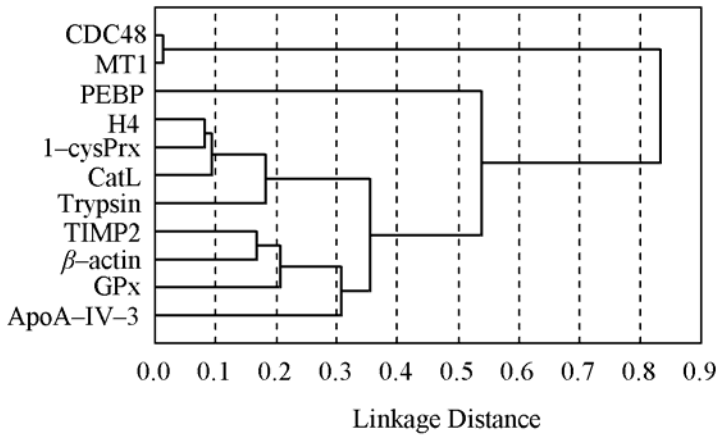


**Fig. 5.1.5.** Joining tree plot of all identified proteins based on regulation factors (single linkage distances using 1-Pearson *r* as the distance measure, unweighted pair-group average).

One of the most important mechanisms of Cd toxicity relates to this metal's ability to replace zinc (Zn) in several proteins, rendering them inactive, ranging from MTs (López-Barea and Gómez--Ariza, 2006) to the anti-oxidant enzyme superoxide dismutase (Bauer et al., 1980) and the nuclear DNA-binding repair enzymes and other zinc finger-class proteins (e.g. Asmuß et al., 2000). It as long been discussed the link between these mechanisms and Cd-induced apoptosis (see, e.g., Hamada et al., 1997 and Risso-de Faverney et al., 2001). Holme and co-workers (2007) found that apoptosis induced by B[a]P to be been linked to ROS and DNA-binding B[a]P metabolites but also that the mechanisms may vary from cell type and contaminant concentration. Both 1-cysPrx and GPx are known to protect cells from apoptosis by scavenging oxidative radicals, therefore reducing oxidative stress (Gouazé et al., 2002; Manevich et al., 2002; Pak et al., 2002). At the injected doses Cd and B[a]P triggered different antioxidative stress responses, generating an antagonistic response during co-exposure: whereas Cd promoted 1-cysPrx upregulation, B[a]P upholded GPx upregulation without affecting 1--cysPrx. The combination of both xenobiotics, however, was observed to reduce the induction of both antioxidant enzymes, which likely exposed cells to increased oxidative stress.

Cathepsin L is a protease specialized in the degradation of cellular and intercellular structural proteins. The activity of this protease has been positively related to apoptosis (Fujimoto et al., 2002). Cathepsins, like caspases, are cystein proteases of the papain family. They are thought to "leak" from lysosomes during apoptosis, contributing to the regulation of caspase-independent apoptosis via the lysosomes-mitochondrial pathway (see Chwieralski et al., 2006 for a review). Cathepsin L has also been found a chief lysosomal protease in cytochrome c-independent apoptosis by activation of caspase-3 in human cell lines (Hishita et al., 2001). It is noteworthy that Cd has already been found capable of triggering caspase-independent apoptosis in normal human lung cells, by translocation of apoptosis induction factor (AIF) from the mitochondria to the nucleus (Shih et al., 2003). Even though the 2DE procedure failed to detect caspases or their precursors, our findings suggest that CatL may be involved in Cd-induced apoptosis and that Cd and B[a]P had, in this study, an antagonist effect on the regulation of this enzyme. Interestingly, apoMT has been found to be rapidly degraded by cathepsins

(including CatL), although metals bound to MT reduce susceptibility of hydrolysis, presumably by blocking the cysteine residues where cathepsins act (Min et al., 1992). It may be suspected that MT levels in Cd-treated livers may not reflect MT expression alone, but also that of cysteine proteinases.

The most striking combination effect observed between Cd and B[a]P in terms of protein expression was related to the observed upregulation of TIMP2, β-actin and ApoA-IV3. The differences between the regulation patterns of these proteins in Cd + B[a]P-treated fish and individuals treated with the isolated toxicants may aid explaining the reduced apoptosis and lack of lipid vacuolation in the livers of fish subjected to Cd + B[a]P. Although it has been sustained that β-actin, a key element of cystoskeletal microfilaments, is necessary to PCD, some authors found that its transcription is not necessarily linked to apoptosis and that, in some cases, it may actually fall (Naora and Naora, 1996). These observations might contribute to explain the reduction in apoptosis in fish treated with Cd + B[a]P, where β-actin upregulation was observed. On the other hand, TIMPs are known apoptosis inhibitors by decreasing metalloproteinase activity (Barasch et al., 1997; Murphy et al., 2002). The reduced hepatocyte apoptosis observed in individuals treated with Cd + B[a]P may thus be partially explained by TIMP upregulation. Apolipoproteins such as ApoA-IV3 play a role in lipid transport as structural components of lipoprotein particles, cofactors for enzymes and ligands for cell--surface receptors. Recently, Apo-IV has been discovered to reduce oxidative stress-driven apoptosis by modulating the glutathione redox status (Spaulding et al., 2006), which may have contributed to the reduced induction of apoptosis in fish co-exposed to Cd and B[a]P. Moreover, Apo-IV upregulation has been observed to promote lipid efflux from cells (Fournier et al., 2000), which may have been responsible for the depletion in intracellular lipid storage observed in the Cd + B[a]P combination treatment. Conversely, Cd- and B[a]P-treated livers depicted a moderate downregulation of Apo-IV that may have promoted lipid vacuolation. Trypsin was another protein upregulated by the combination treatment. Inhibitors of trypsin-like serine proteases, for instance, have been found to induce caspase-linked apoptosis (Murn et al., 2004). Conversely, excessive loads of trypsin and other serine proteases have been found to induce apoptotic-like cell death (Williams and Henckart, 1994). Although much information is still lacking on the exact role of serine proteases in PCD, some authors have suggested that they may either take part in a caspase-independent apoptotic pathway or enhance caspase-induced apoptosis (Stenson-Cox et al., 2003). The present work does not allow further enhancement on the role of trypsin in programmed cell death, however, upregulation of this enzyme in the livers of Cd + B[a]P-treated animals suggests a synergistic effect between the two xenobiotics occurred at the tested doses.

The enforced combination treatment did not reveal any synergistic effects between the two contaminants for phosphatidylethanolamine-binding protein and histone H4. Phosphatidylethanolamine-binding proteins (PEBPs) display several functions, including lipid binding, neuronal development, serine protease inhibition, and the regulation of several stress--involved cellular signalling processes such as the MAPK (mitogen-activated protein kinase) and the NF-kB (nuclear factor kappa binding) pathways. PEPBs are known to have an anti-PCD role by

protecting the membrane integrity during apoptosis, preventing the phospholipid "flip-flop" through which the phospholipid phosphatidylethanolamine is externalized to the outer face of the plasmatic membrane (Wang et al., 2004). Cadmium, isolated and, more significantly, combined with B[a]P, caused a downregulation of PEBPs that was not observed in the B[a]P treatment. Still, the regulation pattern observed for this protein does not clearly relate to the observed yields of apoptosis attained with the applied doses of Cd and B[a]P. It is likely that Cd-induced downregulation of this protein may have facilitated PCD. Conversely, an antagonistic effect between Cd and B[a]P was observed in H4 expression. Histones are fundamental proteins during early-stage apoptosis to make chromatin hypercondensation possible so that, unlike during cell division, heterochromatin looses its structure to permit the binding of endonucleases (Robertson et al., 2000). Up- or downregulation of histones is associated to cell proliferation and cell cycle arrest, respectively. Histone upregulation in the livers of Cd-treated fish could indicate a more advanced stage of tissue regeneration than in B[a]P-treated fish, which is not entirely confirmed histologically, possibly due to the short-term incubation and to the relatively low doses of the injected xenobiotics.

The present work has shown that *in vivo* incubation of teleost livers with low, sublethal, doses of Cd and B[a]P may produce a fast-acting response mechanism by promoting apoptosis and infiltration of defence and stem cells to support tissue regeneration. Although the similarity of these responses can be evaluated histo and cytologically, the biochemical machinery responsible for these responses may differ. Furthermore, without being responsible for lethality or even a considerable loss of hepatic integrity, the combination of the two substances very significantly impaired the liver's defences. Still, it must be noted that much research is yet needed to disclose the complexity of contaminant interactions in the vertebrate liver, regarding, e.g., transmembrane transporters involved in metabolite excretion like the ATP-binding cassette (ABC) transporters or the cell or the signalling pathways that control tissue PCD and tissue regeneration by stem cells and macrophage proliferation. It would also be of importance to explore differential exposure routes and multiple doses, as well as different incubation or exposure periods, which are likely to change the mechanisms and magnitude of xenobiotic interactions. In spite of its limitations caused especially by gaps in peptide databases, proteomics proved to be a relevant tool to identify some of the differentially expressed proteins that account for the dissimilar biochemistry of Cd- and B[a]P-induced apoptosis, anti-oxidative stress and tissue recovery responses without the need of a complete knowledge on the mechanisms of toxicity. Regardless of the usefulness of proteomics in the search for new potential biomarkers in toxicological studies (Monsinjon and Knigge, 2007), the present findings showed that "omics" screening techniques are of relevance when breaking way at understanding the fundamental mechanisms of toxicity. From the present results, it may also be inferred that caution is mandatory when employing common biomarkers, such as MT induction, histopathology and detection of apoptosis in pharmacological and toxicological studies when different classes of contaminants are involved since interaction effects may mask the responses to individual contaminants.

**Acknowledgements**

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P.M. Costa is supported by a FCT PhD grant (SFRH/BD/28465/2006). The authors thank for their support: Pedro Pousão (Estação Piloto de Piscicultura, INRB/IPIMAR-Cripsul); José Alhama Carmona, Carlos A. Fuentes Almagro and Ricardo Fernández Cisnal (Universidad de Córdoba); Lia Ascensão, Luísa Mota and Francisco Carrapiço (Faculdade de Ciências da Universidade de Lisboa).

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# Chapter 6. Integrative perspectives on biomarkers and indicators of toxicity and general conclusions

## 6.1. Can the integration of multiple biomarkers with sediment geochemistry solve the complexity of sediment risk assessment? A case study with a benthic fish[†]


### Abstract

Estuarine sediments are complex contaminant matrices as a consequence of their intricate chemical and physical characteristics and the probable presence of multiple classes of contaminants, whose sorption/desorption cycle is determined by many environmental factors. Surveying the toxicological alterations imposed to organisms by such media for ecological risk assessment (ERA) purposes often results in findings that are either difficult to interpret or even contradictory to acknowledged theory on biomarkers and other indicators of exposure. In order to contribute to the understanding of the biological changes caused by exposure to contaminated estuarine sediments and to evaluate bioassay-based methodologies to toxicity and the significance of the biomarker approach, this work summarizes and integrates the highlights of a series of assays performed in the laboratory and *in situ* with a benthic fish (the Senegalese sole). Juvenile soles were exposed to sediments collected from an estuary acknowledged to be low to moderately contaminated by metallic and organic xenobiotics (the Sado River estuary, W Portugal), which was complemented by the physico-chemical characterization of sediments. Three stations were surveyed, a clean (reference) plus two contaminated, in two distinct seasons: fall 2006 and spring 2007. Several effects and responses to contamination (herewith termed "biomarkers") were surveyed, reflecting different levels of biological sub-individual organization: from liver histopathology and genotoxicity to more advanced "omic" techniques, namely toxicogenomics and proteomics. The integration of data following multivariate statistics and an indice-based biomarker evaluation showed that the combination of multiple classes of contaminants with the overall levels of contamination greatly diminished the specificity of classical biomarkers such as CYP1A and metallothionein induction; however, both could at some extent segregate exposure to contaminated and uncontaminated sediments. On the other hand, biomarkers reflecting lesions, namely histopathological and genotoxicity biomarkers, tend to provide more consistent evidence of exposure to contaminated media, regardless of season, assay type, sampling time and even of intricate biological processes such as the effects of co-exposure to multiple classes of substances and the metabolic unbalancing resulting from the initial phase of exposure, for which the deregulation of many baseline metabolism enzymes constituted evidence. Assets and handicaps of the multiple biomarker sets surveyed and their adequacy of predictive and mechanistic environmental toxicology are debated.


[†] Costa et al. (*submitted*).

**Key-words**

Biomarkers; Bioassays; Sediment quality guidelines; Ecological risk assessment; *Solea senegalensis*; Sado Estuary

## 1. Introduction

The need to reconcile socio-economical activities with environmental quality deems of great importance to develop effective ecological monitoring of preferential areas for human settlement. Amongst these, estuaries are particular cases of concern due to their ecological importance, complexity and, most frequently, high level of anthropogenic pressure. Estuaries are subjected to multiple sources of different kinds of pollutants which, concerning contamination of the water bodies, tend to be trapped in sediments, sorpted to fine particles and organic matter. Estuarine sediments can act, therefore, as reservoirs of contaminants that under certain circumstances may be released back to the water column, rendering them more readily available to the biota and food chains, on top of which lie human populations (see Chapman, 2007, for a review). Therefore, assessing sediment quality, of which toxicity testing is one of the lines-of-evidence (LOEs), is a key element in ecological risk assessment (ERA) strategies for these ecosystems. One can refer, for instance, to the sediment quality triad concept proposed by Long and Chapman (1985), which comprises the surveys for sediment contamination, ecological changes to benthic fauna and toxicity testing to develop effective ecological risk assessment strategies for aquatic sediments.

Testing the toxicity of estuarine sediments, however, is a challenge to environmental toxicologists, in part due to the geochemical complexity of estuarine sediments (which affects contaminant speciation and bioavailability); and in part caused by the likely existence of multiple types of xenobiotics which may mask cause-effect relationships by within-organism interaction effects. To these constraints can be added the complex biological effects and responses to contamination. The biomarker approach has been widely employed in ERA, for both predictive and mechanist toxicological studies. Biomarkers, in a practical term, are considered to reflect “early warnings” to the potential adverse effects caused by xenobiotics to organisms (refer to van der Oost et al., 2003 for a review on the use of biomarkers in aquatic organisms for ERA). In fact, the archetypal definition for “biomarker” in environmental research, as defined by van Gestel and van Brummelen (1996), stand for any sub-individual level alterations resulting from exposure to a given substance. There is a constant need to search and validate adequate biomarkers in toxicity testing for ecotoxicological studies, with especial respect to contaminant mixtures. In the past decade, the novel “omic” approaches (transcriptomics/toxicogenomics, proteomics and metabolomics) introduced new concepts for the simultaneous screening of multiple responses to toxicity and are providing much novel information on the search for novel biomarkers and on the biological processes triggered by

exposure (Snell et al., 2003; Monsinjon and Knigge 2007).

The biomarker approach combined with bioassay techniques has been regarded as an efficient and cost-effective methodology to assess toxicity from environmental contaminants (Lam and Gray, 2003). With respect to testing methodologies, it has been recognized that laboratory bioassays eliminate the variability from environmental confounding factors, therefore probably permitting a more clear-cut relationship between contamination and toxicity; however, it is also acknowledged that these assays tend to either underestimate or overestimate toxicity (Martín-Díaz et al., 2004). Nevertheless, little research is dedicated to the direct comparison between laboratory and *in situ* (field) bioassays with the purpose of ERA on aquatic sediments, even though the adequate choice of testing procedures is yet another pillar of toxicity assessment.

The study area, the river Sado estuary (western Portugal) is the second largest estuarine basin in Portugal (second only to the Tagus estuary, where the capital, Lisbon, is settled), with an approximate area of 240 $km^2$. Although a considerable portion of the estuary is environment-protected, the area is long subjected to many forms of human usage and alteration, many of which are sources of pollutants that are discharged into the basin, of what is considered one of the most important industrial areas in Portugal. The area includes the city of Setúbal (≈ 100,000 inhabitants) which is served by an important commercial harbour and a dense heavy-industry belt that includes chemical plants, a paper mill, a thermoelectrical unit, mineral ore deployment facilities and a large shipyard complex (Fig. 6.1.1). The estuary is also very important for local fisheries, tourism, mariculture and upstream agriculture from which runoffs likely carry pesticides and fertilizers to the estuary. Reinforcing the need to apply effective environmental management and conservation policies, there lays the need to protect the only surviving estuarine population of the bottlenose dolphin (*Tursiops truncatus*) in Portugal (see Caeiro et al., 2009, for an ecological risk assessment strategy for the estuary). For all these reasons, several works appeared to assess the levels of contamination of the estuary and their potential risk to the biota. Although in global terms the estuary can be regarded as moderately contaminated, some sections, especially those located near industrial areas and the lower estuary revealed levels of concern for many contaminants, both organic and inorganic, with adverse toxicological consequences to organisms being found in recent surveys (e.g. Neuparth et al., 2005). Besides the contamination of the water body from urban and industrial origins and subsequent contaminant trapping in sediments, it is believed that most metallic contamination is driven by river transportation, since the Sado crosses an important pyrite mining region (Cortesão and Vale, 1996). Endocrine disruptor compounds (EDCs) have also been recently surveyed in the estuary, with the results revealing globally low levels and likely insufficient to cause adverse effects to organisms (Ribeiro et al., 2009), with similar results being found for mercury (Lillebø et al., 2010).

The Senegalese sole (*S. senegalensis*, Kaup 1858; Teleostei: Pleuronectiformes) is a common soleid in the estuary (that acts an important nursery ground for this and other piscine species) exhibiting a pronounced seasonal variation in its distribution with a tendency to occupy the inner areas of the estuary, preferring sandy-muddy floors where it feeds on small invertebrates (Cabral, 2000).

Levels of concern for organic and inorganic contaminants were found in these areas (Caeiro et al., 2005; Lobo et al., 2010). The species has a considerable economical value for local fisheries, as in SW Europe and benefits from a good potential for aquaculture activities (see Diniz et al., 1999), which facilitates access to hatchery-brood animals for research purposes. In the past decade, several bioassay-based ecotoxicological studies have appeared using this species, ranging from baseline toxicological surveys (e.g. Arellano et al., 1999; Costa et al., 2010b) to environmental monitoring (Jiménez-Tenorio et al., 2007) and even toxicological studies of relevance for aquaculture (Osuna--Jiménez et al., 2009). The rising number of studies being enforced using the species as test organism may be providing it with the potential for the monitoring of aquatic sediments that other flatfish species are recognized with, such as the flounder (*Platichthys flesus*) in Northern Europe.

This work will review and integrate previous data obtained for sediment contamination profiles with biomarker analyses performed on *S. senegalensis* exposed to sediments from the Sado estuary. It is intended to assess the constraints and assets of laboratory and field bioassays for the assessment of sediment risk and the adequacy of the multi-level biomarker approach employed and to contribute to a better understanding of the complex biological mechanisms of response to sediment--bound contaminants and their mixtures. The present study will mainly focus on the biomarker (or potential biomarker) significance and validation to determine sediment risk, under a comparative approach between clean and contaminated sediments.

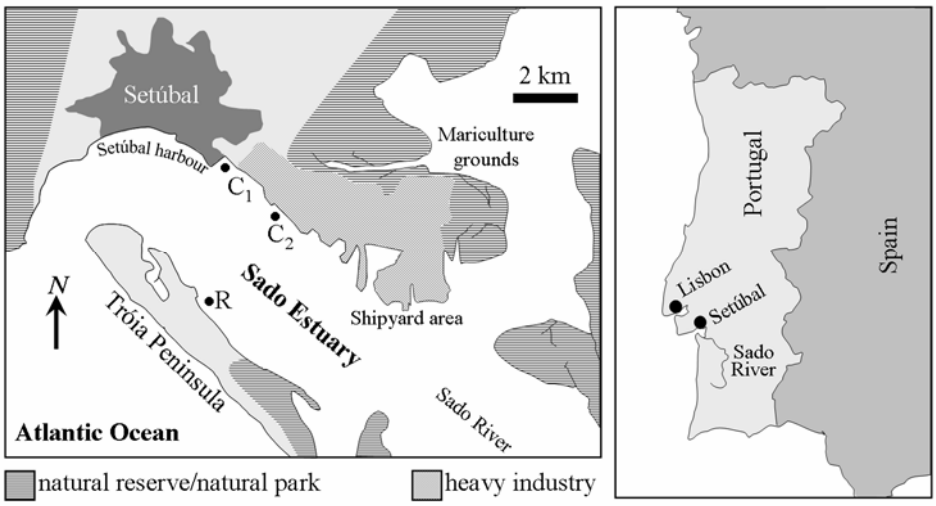


**Fig. 6.1.1.** Map of the study area marking (•) the three sites under survey: R (reference), and contaminated ($C_1$ and $C_2$).

## 2. Methods and materials

### *2.1. Study sites*

Three sites of the Sado Estuary with potentially different characteristics were chosen for the survey (Fig 6.1.1), according to previous studies that characterized the area for sediment

contamination and ecological risk (Caeiro et al., 2005; Neuparth et al., 2005; Costa et al., 2008a). The reference site (R) is located off the Tróia peninsula environmental protected area and is the farthest from pollution sources. The contaminated sites $C_1$ and $C_2$ were chosen for their distinct characteristics: site $C_1$ is located at the Setúbal commercial harbour, in an area of low hydrodynamics and high water residence time while site $C_2$ is located off the industrial belt, between the city and a large shipyard facility.

*2.2. Experimental procedures*

Two series of time-displaced 28-day bioassays with juvenile hatchery-brood *Solea senegalensis* (all from the same cohort) as test species were performed. A first series was done under controlled laboratory conditions with sediments from the three sites collected in November 2006 (termed "fall" assay). The following series comprised simultaneous laboratory and field (*in situ*) tests during April-May 2007 ("spring" assay). Sediment samples were collected with a grab and divided for the laboratory assays and for contaminant determination.

The laboratory assays were performed in duplicate in white 15-L capacity polyvinyl tanks with blunt edges in which were allocated 2 L of fresh sediment plus 12 L of clean seawater. The parameters were similar between fall and spring assays, and are described in detail elsewhere (Costa et al., 2008b; 2011a). In brief: temperature and photoperiod were held constant at ≈ 18ºC and 12:12 h, respectively. Salinity, pH and ammonia were restrained at approximately 33, 8 and 2 $mg.L^{-1}$, respectively, by means of a weekly 25 % water volume change. A recirculation arrangement and constant aeration were adapted to the test tanks with the flows being adjusted to minimize resuspension. Dissolved $O_2$ was ≈ 50%. Twenty (spring) or twenty-four (fall) fish (standard length class ≈ 60-70 mm) were allocated per tank. Weather conditions at the study area did not make possible to perform *in situ* assays during fall. Field assays were done at the following spring with submerged cages (90 × 90 × 30 cm) lined with a 5 mm plastic mesh. Each cage was divided into two compartments, each being regarded as a replicate. Twenty animals were allocated in each compartment. The cages were placed over the bottom by scuba diving, ensuring contact with the sediment, at depths ranging between 7 and 9 m. For all assays, at days 14 ($T_{14}$) and 28 ($T_{28}$), five to six fish were retrieved and sampled for biological analysis. Ten to twelve fish collected directly from the rearing tanks were also sampled ($T_0$ fish) to establish the baseline condition of the animals.

*2.3. Sediment characterization*

From the sediments collected for the fall assays the metalloid arsenic (As) and the metals cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) nickel (Ni), and zinc (Zn) were determined by inductively coupled plasma mass spectrometry (ICP-MS) after acid digestions in Teflon vials, according to Caetano et al. (2007), to which ere added the non-meal selenium (Se) plus the metals

cobalt (Co) and manganese (Mn), quantified by the same procedure, to which was added mercury (Hg), determined by atomic absorption spectrometry (AAS) according to Costley et al. (2000). Element quantifications were validated by analysis of the reference sediments MESS-2and PACS-2 (National Research Council, Canada) and MAG-1 (US Geological Survey, USA) by the same procedures. The measured values were found to be within the certified range.

Organic contaminants were determined in dried samples of sediments collected at both fall and spring assays, namely polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and the pesticide dichloro-diphenyl-trichloroethane (DDT). Sediment PAHs were determined by gas chromatography-mass spectrometry (GC-MS) as described by Martins et al. (2008), after Soxhlet-extraction with an acetone+hexane mixture, with seventeen 3- to 6-ring PAHs being quantified. Organochlorines (18 PCB congeners and DDT plus its main metabolites, meaning *pp*'DDT plus *pp*'DDD and *pp*'DDE) were quantified by GC with electron capture detection (GC-ECD) following Soxhlet-extraction with *n*-hexane and fractioning in a chromatographic column as described by Ferreira et al. (2003). The procedures were validated by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA). The obtained values were found within the certified range.

Sediment redox potential (Eh) was measured immediately after sediment collection. Sediment fine fraction (FF, particle size < 63 μm) was determined after sediment disaggregation with pyrophosphate and hydraulic sieving. Total organic matter (TOM) was extrapolated from carbon loss--on-ignition (LOI) at 500 ± 50 ºC. The TOM and FF levels are given as a percentage of sediment total dry mass. Further details on the procedures described above are given by Costa et al. (2008a; 2008b).

### *2.4. Biological analyses and biomarker approach*

The data hereby integrated is described in a series of previous studies (Table 6.1.1). The biomarker approach has been subdivided in two major categories: biomarkers of exposure and biomarkers of effect (Martín-Díaz et al., 2004). The details on the techniques are described elsewhere. In brief: genotoxicity biomarkers were analysed in fall and spring assays by means of the alkaline version of the single-cell gel electrophoresis ("comet") assay to determine total DNA strand breakage (Singh et al., 1998) and through the analysis of erythrocytic nuclear abnormalities (ENA) using a fluorescence microscopy method (Costa and Costa, 2007), both in peripheral blood. Histopathological analysis was achieved by obtaining individual liver histopathological conditions indices ($I_h$), adapted from Bernet et al. (1999) that integrated several potential histological biomarkers such as necrosis, hepatocellular alterations and the presence of intraplasmatic inclusions (termed degeneration) in hepatocytes (e.g. lipid vacuoles and hyaline degeneration). The indices are obtained for each individual and are estimated based on the attribution of an importance factor, or weight, to each histopathological change. The weight values range between 1 (low severity, such as inflammatory changes) to 3 (high severity such as necrosis). Details on the surveyed histological traits and their

significance are debated elsewhere (Costa et al., 2009b, 2011). The $I_h$ indice shall be throughout regarded as a single "biomarker". Since the most representative alterations correspond to lesions (Costa et al., 2009b, Costa et al., 2011), histopathological alterations were included, as for genotoxicity, in the biomarkers of effect category.

Two (potential) biomarkers of exposure were analysed in both sets of assays: cytochrome P450 1A and thiolic protein (metallothionein-like) induction. The induction of CYP1A (cytochrome P450 1A) protein in liver was determined by enzyme-linked immunosorbent assay (ELISA) in the microsomal fraction of homogenates, according to Nilsen et al. (1998) in the fall assays, and by quantitative real-time reverse transcription polymerase chain reaction (qRT-RT PCR) in the spring assays, after *Solea senegalensis* sequencing of the CYP1A1 gene (GenBank accession GU946412) and design of sequence-specific primers (Costa et al., submitted, Section 4.3). Metallothionein-like protein induction in the liver was determined by differential pulse polarography with a static mercury drop electrode (DPP-SMDE), as described by Costa et al. (2008a) and by qRT-RT PCR as aforesaid (Costa et al., submitted, see Section 4.3), after MT1 (metallothionein isoform 1) gene sequencing and specific primer design (GenBank accession GU946410). The analysis of hepatic MT1 and CYP1A1 transcription is part of a toxicogenomic approach (Costa et al., submitted, Section 4.3) that comprised also the analysis of the caspase 3 apoptosis-related gene (CASP3, GenBank accession HQ115741), catalase (CAT, GU946411), Glutathione peroxidase 1 (GPx, HM068301) all first-time sequenced for their respective cDNAs, to which was added the heat-shock protein 90 kDa alpha (HSP90AA/$\alpha$, AB367526), first characterized by Manchado and co-workers (2008) for *S. senegalensis*. The changes in gene transcription were estimated taking $T_0$ animals as the calibrator group, from the relative transcription ratios estimated according to Pffafl (2001). The toxicogenomic approach performed on spring-tested soles was complemented by the analysis of hepatocyte apoptosis through the TUNEL (TdT-mediated dUTP-X nick end labelling) reaction on paraffin-embedded sections. The hepatic apoptotic indice (AI) is expressed as the number of TUNEL-positive cells per section $mm^2$. Complementarily, a proteomic survey was performed on the livers of fish tested in the spring assays (assayed in the laboratory and *in situ*) to screen changes in cytosolic proteins' regulation patterns, though the combination of two-dimensional electrophoresis (2DE) with tandem mass spectrometry techniques (Costa et al., submitted, see Section 4.4). As previous, $T_0$ fish were considered as the calibrator group in order to provide the protein regulation variation coefficients. For simplification (and analytical) purposes, and due to the potentially higher specificity of these responses to a contaminant group or group of contaminants, all biomarker responses related to gene expression or protein induction are allocated in the biomarkers of exposure category.

**Table 6.1.1.** Biomarker approach summary per assay type and respective data sources.

| Assay season | Assay type | Biomarker type | Biomarker | Target | Method | Data origin |
|---|---|---|---|---|---|---|
| *Fall* | Laboratory | Exposure | Total DNA strand breakage | Whole-blood | Comet assay | Costa et al. (2008b) |
| | | | Chromosomal clastogenesis | Mature erythrocytes | ENA sorting | Costa et al. (2008b) |
| | | | Histopathological biomarkers | Liver | Histopathological indices | Costa et al. (2009b) |
| | | Effect | MT induction | Liver | DPP-SMDE | Costa et al. (2009a) |
| | | | CYP1A induction | Liver | ELISA | Costa et al. (2009a) |
| *Spring* | Laboratory | Exposure | Total DNA strand breakage | Whole-blood | Comet assay | Costa et al. (2010c) |
| | | | Chromosomal clastogenesis | Mature erythrocytes | ENA sorting | Costa et al. (2010c) |
| | | | Histopathological biomarkers | Liver | Histopathological indices | Costa et al. (2011) |
| | | Effect | MT induction | Liver | qRT-RT PCR | Costa et al. (submitted) |
| | | | CYP1A induction | Liver | qRT-RT PCR | Costa et al. (submitted) |
| | Field | Exposure | Total DNA strand breakage | Whole-blood | Comet assay | Costa et al. (2010c) |
| | | | Chromosomal clastogenesis | Mature erythrocytes | ENA sorting | Costa et al. (2010c) |
| | | | Histopathological biomarkers | Liver | Histopathological indices | Costa et al. (2011) |
| | | Effect | MT induction | Liver | qRT-RT PCR | Costa et al. (submitted) |
| | | | CYP1A induction | Liver | qRT-RT PCR | Costa et al. (submitted) |

*2.5. Statistics and integration of data*

The integration of sediment contamination data was based on the sediments' potential impact to cause adverse biological effects, estimated by calculating the *PEL* quotient (*PEL-Q*) based on the published guideline values for coastal waters, namely the threshold effects level (*TEL*) and the probable effects level (*PEL*), according to MacDonald et al. (1996). The *PEL-Q* indice was obtained for each contaminant or class of contaminants (depending on available data) according to the formula described by Long and MacDonald (1998):

$$PEL - Q_i = \frac{C_i}{PEL} \quad [1]$$

The *PEL* value is the guideline value for the contaminant *i* and $C_i$ the effective measured concentration of the contaminant in the sediment. The Sediment Quality Guideline Quotient indice (*SQG-Q*), developed to compare sites impacted by mixtures of contaminants, was calculated for each sediment (according to Long and MacDonald, 1998) as:

$$SQG - Q = \frac{\sum_{i=1}^{n} PEL - Q_i}{n} \quad [2]$$

Where $PEL\text{-}Q_i$ is the indice deriving from formula [1] for the $i^{th}$ contaminant and *n* the number of surveyed contaminants. The three sites were scored according to their overall potential of observing adverse biological effects according to the criteria: $SQG\text{-}Q < 0.1$ - unimpacted; $0.1 \leq SQG\text{-}Q < 1$ - moderately impacted; $SQG\text{-}Q \geq 1$ - highly impacted (MacDonald et al., 2004).

For statistical purposes, all biomarker data were converted into variation coefficients (VC) relatively to $T_0$ fish (which in essence reflect the rearing conditions) in order to obtain a relative measure of change imposed by laboratory and field exposures to the sediment of all surveyed sites, reference included. Average variation coefficients were obtained for biomarkers of effect and responses for comparison purposes. The Mann-Whitney *U* test was employed for pairwise comparisons between experimental conditions. The integration of *SQG-Q*s with biomarker data was achieved by factor analysis with extraction by principal components. Cluster analysis based on the 1-Pearson correlation statistic *r* was done to survey links between biological responses and effects. All statistics were performed with the software Statistica (Statsoft Inc.). A significance level of $\alpha = 0.05$ was considered for all analyses.

In order to assess the consistency of each measured response and effect to detect significant differences between fish exposed to contaminated and clean (reference) sediments, a biomarker consistency indice ($B_i$) was developed, being defined as:

$$B_i = \frac{\sum_{j=1}^{n} s_j}{n} \quad [3]$$

Being $B_i$ the biomarker consistency indice for the $i^{th}$ response or effect, $n$ the number of cases in the experimental subset under scope and $s_j$ the "score" for the $j^{th}$ case, which can attain the value of -1 if a significant decrease in the response was observed (Mann-Whitney $U$, $p < \alpha$); 1 if a significant increase was observed or 0 if no statistical differences between exposure to contaminated and reference sediments were detected.

## 3. Results

### *3.1. Sediment characterization*

The reference sediment R remained the least contaminated sediment from fall to spring. However, the overall contamination of sediment $C_2$ increased, due to higher levels of metals and arsenic (Table 6.1.2). Still, sediments from site $C_1$ contained the highest load of organic matter and particle fine fraction and sediment $C_2$ was found to be the least anoxic, at both seasons. Although total Hg was not determined at the fall assays, the contribution of this metal to the overall *SQG-Q* of sediments $C_1$ and $C_2$ was low (even though in both cases total Hg surpassed *TEL* and *PEL* levels, respectively), since the *SQG-Q* for total contamination without the consideration of total Hg (for the spring assays) was 0.31 and 0.24 for sediment from sites $C_1$ and $C_2$, respectively. Although the differences between the two contaminated sediments diminished from fall to spring even though site $C_1$ remained the most contaminated (especially by metals), followed by site $C_2$ (the most contaminated by organic xenobiotics). The reference sediment revealed low contamination levels, with decreased *SQG-Q*s from fall to spring. The essential metals copper plus the metalloid arsenic were consistently the elements of most concern at both seasons, with, inclusively, Cu and Zn reaching *TEL* levels in site R in the fall.

For both batches of sediments the phenanthrene/anthracene and fluoranthene/pyrene ratios were found to be > 1 and < 10, respectively, which indicates that the PAHs are mostly of pyrolytic origin (combustion-derived) and not petrogenic (Budzinski et al., 1997). At both seasons, the 4-ring PAHs fluoranthene (in $C_1$ and $C_2$) plus pyrene and benzo[a]anthracene (only in $C_2$ in the fall) presented levels of concern, to which is added the 5-ring dibenzo[a,h]anthracene. In the spring, the 5--ring, known carcinogen, benzo[a]pyrene reached *TEL* levels in the two contaminated sediments. Overall, the PAHs concentrations in sediments $C_1$ and $C_2$ were higher in the spring comparing to the previous fall season, with the inverse being observed for the reference sediment. Four-ring PAHs were always the most representative substance of the class, ranging approximately between 40-50% of total PAHs.

**Table 6.1.2.** Sediment characterization for the sediments of the three surveyed sites (R, reference plus $C_1$ and $C_2$, contaminated - referred to as A, B and C, respectively, in Chapter 3) at the two series of assays: laboratory (fall 2006) and simultaneous laboratory and field (spring 2007). The *PEL* and *TEL* sediment quality guidelines were retrieved from MacDonald et al. (1996).

| | | | Fall† | | | Spring‡ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *Site* | **R** | **$C_1$** | **$C_2$** | **R** | **$C_1$** | **$C_2$** | | |
| | | TOM (%) | 3 | 12 | 8 | 2 | 10 | 7 | | |
| | | FF (%) | 37 | 98 | 76.8 | 23 | 96 | 76 | | |
| | | Eh (mV) | -233 | -290 | -316 | -140 | -300 | -312 | *SQGs* | |
| Contaminant | | | | | | | | | *TEL* | *PEL* |
| Element (μg g$^{-1}$ sediment dw) | Non-metal | Se | - | - | - | 0.27±0.01 | 1.21±0.02 | 0.80±0.02 | *NG* | *NG* |
| | Metalloid | As | 7.25±0.15* | 27.43±0.55* | 12.38±0.25* | 5.20±0.10 | 23.98±0.48* | 20.69±0.41* | 7.24 | 41.60 |
| | Metal | Cd | 0.04±0.00 | 0.22±0.00 | 0.15±0.00 | 0.06±0.00 | 0.26±0.01 | 0.29±0.01 | 0.68 | 4.21 |
| | | Co | - | - | - | 3.37±0.07 | 13.94±0.28 | 9.43±0.19 | *NG* | *NG* |
| | | Cr | 24.20±0.48 | 76.33±1.53* | 21.85±0.44 | 18.14±0.36 | 80.73±1.61* | 51.70±1.03 | 52.3 | 160 |
| | | Cu | 22.57±0.45* | 167.32±3.35** | 41.18±0.82* | 28.20±0.56* | 172.72±3.45** | 95.31±1.91* | 18.7 | 108 |
| | | Hg | - | - | - | 0.11±0.00 | 0.69±0.01* | 0.71±0.01** | 0.13 | 0.70 |
| | | Mn | - | - | - | 100.75±2.01 | 464.34±9.29 | 362.47±7.25 | *NG* | *NG* |
| | | Ni | 12.97±0.26 | 33.67±0.67* | 9.03±0.18 | 7.31±0.15 | 33.30±0.67* | 20.49±0.41* | 15.9 | 42.8 |
| | | Pb | 23.70±0.47 | 66.49±1.33* | 45.17±0.90* | 18.57±0.37 | 55.19±1.10* | 43.76±0.88* | 30.2 | 112 |
| | | Zn | 147.48±2.95* | 312.23±6.24** | 87.75±1.76 | 72.29±1.45 | 364.83±7.30** | 269.31±5.39* | 124 | 271 |
| | | *SQG-Q* Element | **0.23** | **0.75** | **0.26** | **0.16** | **0.79** | **0.58** | | |
| PAH | 3-ring | Acenaphthylene | 0.24±0.04 | 1.83±0.31 | 1.95±0.33 | 0.79±0.13 | 2.38±0.40 | 2.18±0.37 | 5.87 | 128 |
| | | Acenaphthene | 1.41±0.24 | 9.42±1.60* | 4.19±0.71 | 0.73±0.12 | 12.25±2.08* | 7.83±1.33* | 6.71 | 88.9 |
| | | Fluorene | 1.32±0.22 | 8.70±1.48 | 8.03±1.37 | 1.19±0.20 | 15.33±2.61 | 9.95±1.69 | 21.2 | 144 |
| | | Phenanthrene | 7.96±1.35 | 50.77±8.63 | 54.09±9.20 | 10.28±1.75 | 63.87±10.86 | 59.91±10.18 | 86.7 | 544 |
| | | Anthracene | 1.03±0.17 | 10.60±1.80 | 15.34±2.61 | 2.30±0.39 | 21.00±3.57 | 20.84±3.54 | 46.9 | 245 |
| | 4-ring | Fluoranthene | 18.05±3.07 | 170.80±29.04* | 184.30±31.33* | 23.34±3.97 | 315.71±53.67* | 345.24±58.69* | 113 | 1494 |
| | | Pyrene | 14.66±2.49 | 131.74±22.40 | 171.39±29.14* | 21.51±3.66 | 263.18±44.74* | 286.33±48.68* | 153 | 1398 |
| | | Benzo[a]nthracene | 4.53±0.77 | 64.60±10.98 | 86.52±14.71* | 3.70±0.63 | 81.25±13.81* | 93.99±15.98* | 74.8 | 693 |
| | | Chrysene | 2.20±0.37 | 28.31±4.81 | 37.19±6.32 | 2.35±0.40 | 41.06±6.98 | 46.68±7.94 | 108 | 846 |
| | 5-ring | Benzo[b]fluoranthene | 6.77±1.15 | 60.86±10.35 | 70.25±11.94 | 5.71±0.97 | 98.00±16.66 | 115.97±19.72 | *NG* | *NG* |
| | | Benzo[k]fluoranthene | 4.16±0.71 | 32.21±5.48 | 40.18±6.83 | 2.22±0.38 | 30.76±5.23 | 44.82±7.62 | *NG* | *NG* |
| | | Benzo[e]pyrene | 5.12±0.87 | 56.73±9.64 | 62.76±10.67 | 4.80±0.82 | 74.95±12.74 | 8.96±1.52 | *NG* | *NG* |
| | | Benzo[a]pyrene | 7.56±1.28 | 69.81±11.87 | 85.88±14.60 | 5.42±0.92 | 101.86±17.32* | 126.76±21.55* | 88.8 | 763 |
| | | Perylene | 4.69±0.80 | 86.97±14.79 | 209.16±35.56 | 7.83±1.33 | 96.29±16.37 | 113.47±19.29 | *NG* | *NG* |
| | | Dibenzo[a,h]anthracene | 0.74±0.13 | 7.45±1.27* | 6.99±1.19* | 0.66±0.11 | 13.32±2.26* | 13.93±2.37* | 6.22 | 135.00 |
| | 6-ring | Indeno[1,2,3-cd]pyrene | 4.87±0.83 | 52.44±8.91 | 51.82±8.81 | 5.06±0.86 | 82.06±13.95 | 95.00±16.15 | *NG* | *NG* |
| | | Benzo[g,h,i]perylene | 1.12±0.19 | 39.12±6.65 | 10.44±1.78 | 3.91±0.66 | 51.93±8.83 | 55.85±9.49 | *NG* | *NG* |
| | | *tPAH* | *86.4±14.7* | *882.4±150.0* | *1,100.5±187.1* | *101.8±17.3* | *1,365.2±232.1* | *1,447.7±246.1* | *1,684* | *16,770* |
| | | *SQG-Q* PAH | **0.01** | **0.07** | **0.08** | **0.01** | **0.11** | **0.12** | | |
| Organic (ng g$^{-1}$ sediment dw) | Trichlorinated | 18 | 0.04±0.01 | 0.08±0.01 | 0.09±0.01 | < d.l. | 0.27±0.05 | 0.42±0.07 | *NG* | *NG* |
| | | 26 | 0.05±0.01 | 0.06±0.01 | 0.09±0.01 | < d.l. | 1.80±0.31 | 1.99±0.34 | *NG* | *NG* |
| | Tetrachlorinated | 31 | 0.64±0.11 | 0.19±0.03 | < d.l. | 0.05±0.01 | 0.26±0.04 | 0.34±0.06 | *NG* | *NG* |
| | | 44 | 0.05±0.01 | 0.38±0.06 | < d.l. | 0.03±0.01 | 0.17±0.03 | 0.40±0.07 | *NG* | *NG* |
| | | 49 | 0.04±0.01 | 0.08±0.01 | 0.36±0.06 | < d.l. | 0.13±0.02 | 0.24±0.04 | *NG* | *NG* |
| | | 52 | 0.05±0.01 | 0.12±0.02 | 0.45±0.08 | < d.l. | 0.10±0.02 | 0.44±0.07 | *NG* | *NG* |
| | Pentachlorinated | 101 | 0.04±0.01 | 0.23±0.04 | 1.18±0.20 | 0.03±0.01 | 0.25±0.04 | 0.46±0.08 | *NG* | *NG* |
| | | 105 | 0.03±0.01 | 0.22±0.04 | 0.66±0.11 | < d.l. | 0.26±0.04 | 0.39±0.07 | *NG* | *NG* |
| | | 118 | < d.l. | 1.04±0.18 | 4.92±0.84 | < d.l. | 0.55±0.09 | 0.77±0.13 | *NG* | *NG* |
| PCB | Hexachlorinated | 128 | 0.01±0.00 | 0.08±0.01 | < d.l. | < d.l. | 0.26±0.04 | 0.48±0.08 | *NG* | *NG* |
| | | 138 | 0.12±0.02 | 0.68±0.12 | 2.68±0.46 | 0.14±0.02 | 0.71±0.12 | 1.08±0.18 | *NG* | *NG* |
| | | 149 | 0.11±0.02 | < d.l. | < d.l. | 0.08±0.01 | 0.05±0.01 | 0.30±0.05 | *NG* | *NG* |
| | | 151 | 0.05±0.01 | 0.17±0.03 | 1.15±0.20 | 0.06±0.01 | 0.77±0.13 | 1.14±0.19 | *NG* | *NG* |
| | | 153 | 0.14±0.02 | 0.64±0.11 | 3.39±0.58 | 0.13±0.02 | 0.98±0.17 | 1.28±0.22 | *NG* | *NG* |
| | Heptachlorinated | 170 | 0.07±0.01 | 0.27±0.05 | < d.l. | 0.07±0.01 | 0.20±0.03 | 0.25±0.04 | *NG* | *NG* |
| | | 180 | 0.21±0.04 | 0.61±0.10 | < d.l. | 0.05±0.01 | 0.73±0.12 | 1.22±0.21 | *NG* | *NG* |
| | | 187 | 0.20±0.03 | 0.72±0.12 | < d.l. | 0.14±0.02 | 0.29±0.05 | 0.47±0.08 | *NG* | *NG* |
| | | 194 | 0.03±0.00 | 0.07±0.01 | 0.38±0.06 | 0.01±0.00 | 0.12±0.02 | 0.29±0.05 | *NG* | *NG* |
| | | *tPCB* | *1.87±0.32* | *5.64±0.96* | *15.34±2.61* | *0.80±0.14* | *7.91±1.34* | *11.97±2.04* | *21.6* | *189* |
| | | *SQG-Q* PCB | **0.01** | **0.03** | **0.08** | **0.00** | **0.04** | **0.06** | | |
| DDT | | *pp*'DDD | 0.10±0.02 | 0.28±0.05 | 0.60±0.10 | < d.l. | 0.37±0.06 | 0.71±0.12 | 1.22 | 7.81 |
| | | *pp*'DDE | 0.05±0.01 | 0.27±0.05 | 0.65±0.11 | < d.l. | < d.l. | 0.59±0.10 | 2.07 | 374 |
| | | *pp*'DDT | 0.70±0.12 | 4.39±0.75* | 1.18±0.20 | < d.l. | < d.l. | 1.22±0.21* | 1.19 | 4.77 |
| | | *tDDT* | *0.85±0.14* | *4.94±0.84** | *2.43±0.41* | < d.l. | *0.37±0.06* | *2.52±0.43* | *3.89* | *51.7* |
| | | *SQG-Q* DDT | **0.05** | **0.32** | **0.11** | **0.00** | **0.02** | **0.12** | | |
| | | *SQG-Q* Organic | **0.02** | **0.11** | **0.08** | **0.01** | **0.09** | **0.11** | | |
| | | *SQG-Q* Total | **0.08** | **0.32** | **0.14** | **0.06** | **0.33** | **0.28** | | |
| | | Impact rating | **No impact** | **Moderate** | **Moderate** | **No impact** | **Moderate** | **Moderate** | | |

[*]and [**] indicate substance levels above *TEL* and *PEL* thresholds, respectively.
†From Costa et al. (2008, 2009a, 2009b).
‡From Costa et al. (2010c, 2011).
[-], not determined; < d.l., below detection limit; *N.G.*, no guideline available; *PEL*, probable effects level sediments quality guideline; *SQG*, sediment quality guideline; *TEL*, threshold effects level sediment quality guideline.

Regarding sediment organochlorines, different patterns were observed between the two seasons. While penta- and hexachlorinated CBs where the best represented CBs in sediments from sites $C_1$ and $C_2$ in the fall (trichlorinated in the reference sediment), ranging approximately between 25 and 50%, in the following spring the pentachlorinated compounds lost their prevalence to trichlorinated CBs (≈ 20-30%) in the two contaminated sites. The levels of DDTs were also dissimilar, with sediment $C_1$ presenting the highest concentration in the fall and $C_2$ in the spring. The *pp*'DDT compound was always, however, the most representative DDT.

*3.2. Biomarker approach*

Distinct patterns of biomarker variation coefficients were observed between seasons, assay type and sampling time, besides between exposure to the three surveyed sediments (Table 6.1.3). Exposure to the contaminated sediments $C_1$ and $C_2$ in the laboratory (at either season) caused more significant differences to the response VCs in caged fish assayed in the spring. In addition, in both series of laboratory assays, exposure to the contaminated sediments for 28 days yielded more significantly different responses comparatively to the respective exposure to sediment R. Also, the increase in the VCs is overall more evident for biomarkers of effect than those of exposure, regardless of assay type and season. The most severe DNA damage (given either by TSB or ENA analyses) relatively to initial-state ($T_0$) fish was found in laboratory-exposed animals (with significant differences to R-tested fish also) to sediment $C_2$ (most contaminated by organic pollutants) for 28 days, at both seasons. However, the most severe histopathological changes were observed in the livers of fish exposed in the spring for 28 days to sediments $C_1$ in the laboratory assays and, especially, $C_2$ *in situ* (Fig. 6.1.2). The induction of protein content or gene transcription, on the other hand, revealed variable trends concerning the increase or decrease of responses in all assays, yielding a tendency to downregulate at $T_{14}$ in animals exposed to the contaminated sediments comparatively to $T_0$, and, most notoriously, to R-tested fish in both laboratory and *in situ* bioassays. Hepatocyte apoptosis (measured only in the spring assays) was only significantly increased in laboratory-exposed fish to sediment $C_2$ in relation to the reference treatment whereas in the field assays, a significant decrease was observed at $T_{14}$ in fish exposed to the two contaminated sediments comparing to R-tested soles.

By combining the results from the two season by averaging the VCs for the same surveyed biomarkers of effect (TSB, ENA and $I_h$) and exposure (CYP1A and MT induction, even though these were surveyed by different methods), it was observed that there is a greater contribution to change in relation to $T_0$ animals caused by the biomarkers of effect (Fig. 6.1.3). Factorial analysis on this same data plus the sediment *SQG-Q*s, again combining fall and spring assays, showed that the average variation of the biomarkers of effect correlates best with the sediments' potential to cause adverse effects to organisms with respect to global and *in situ* assay conditions whereas laboratory assays revealed a less clear pattern between variables (Fig. 6.1.4). The factor loadings obtained for the factor analysis main functions F1 (≈ 50-70% total explained variance) and F2 (≈ 20-30% total explained

variance) sustain that the average VC of the considered biomarkers of effect is better correlated to *SQG-Q*s for registering positive values in F1 as the sediment-related variables, especially in field assays (with a loading of 0.91). The average VC for the biomarkers of exposure, on the other hand, provided a significant negative contribution to F1 in the field assays (with a loading of -0.78), which indicates a negative link to the environmental a biomarker of effect variables in the same assays. Regarding the laboratory assays, the average VC for the biomarkers of exposure revealed a more significant contribution to F2 (factor loading = 0.95) with an opposite trend to the biomarker of effect's, similar to the global analysis results. Overall, the loadings should indicate that the variation of the biomarkers of exposure has a significant role in explaining the total variance, however linked to unknown variables.

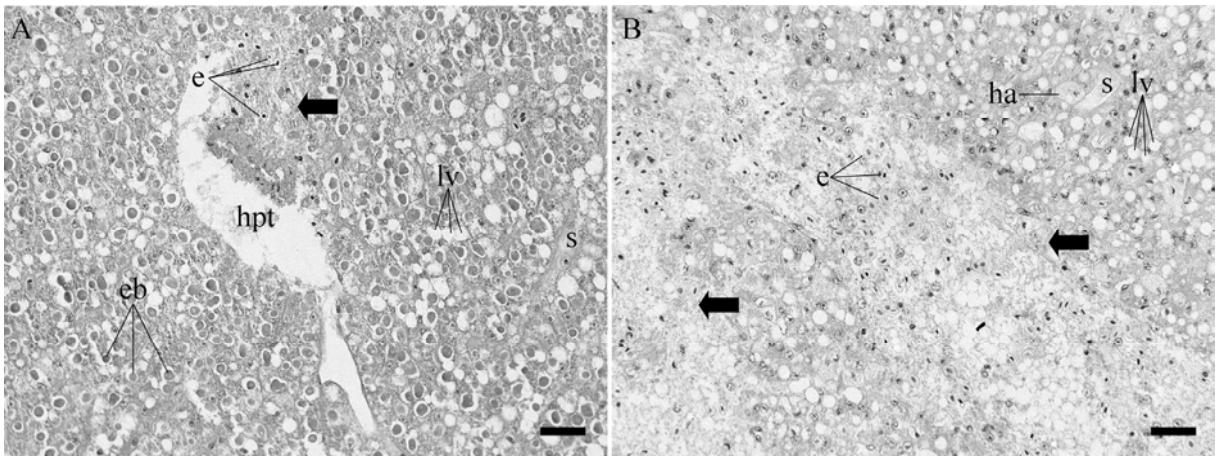


**Fig. 6.1.2.** Representative example of hepatic histopathological alterations observed in fish exposed to the contaminated sediments $C_1$ and $C_2$ exhibiting necrotic foci (arrows). Bouin-Hollande fixative + H&E stain. The presence of erythrocytes (e) inside the foci indicate some extent of haemorrhage spreading from adjacent blood vessels, especially branches of the hepatic portal triad (hpt) A) Hepatic parenchyma of a fish exposed to sediments from site $C_2$ (most contaminated by organic substances) for 28 days during the fall assays (laboratory), from which resulted the most severe histopathological alterations. Besides diffuse lipidosis ("fat" degeneration) these animals revealed diffuse hyaline degeneration of hepatocytes caused by the retention of large eosinophilic bodies (bd) within, resembling in aspect (but not size) Mallory-Denk bodies described in mammals. B) Liver section of a sole exposed to sediment $C_1$ (overall most contaminated) for 28 days *in situ* (spring), depicting severe necrotic diffusion combined with eosinophilic hepatocellular alteration (ha), a lesion normally regarded as pre-neoplasic, and lipidosis (lv). Hyaline degeneration was absent in fish exposed to sediment $C_1$ and $C_2$ in the spring. s) indicates sinusoid vessels.

### *3.3. Global evaluation of biomarker responses*

On Fig. 6.1.5 are indicated the estimates of the $B_i$ indice [3] for each biomarker response, grouped by experimental subset. The results indicate that the biomarkers reflecting lesions (TSB, ENA and $I_h$) were globally more consistent to detect significant differences between exposure to contaminated and clean sediments than protein content and gene transcription responses for both

seasons. However, transcription of the CAT and CYP1A genes were revealed to be more consistent than the transcription of other toxicologically-relevant genes in the spring assays. There were differences in biomarker response consistency in fall and spring assays when grouping sampling time and site: in the fall assays only $I_h$ could consistently distinguish exposure to contaminated from uncontaminated sediments at $T_{14}$ whereas at $T_{28}$ genotoxicity biomarkers also had perfect consistency. Exposure to sediment $C_2$ (mostly contaminated by organic xenobiotics) in the same series of assays also revealed better consistency than tests with $C_1$ sediment (most contaminated by metals) for all biomarkers except CYP1A induction. Regarding the spring assays, biomarker consistency was more variable outside the biomarker of effect group, with some transcript responses yielding opposite trends, such as for HSP90α and GPx in laboratory *versus in situ* and these plus MT1 and CASP3 $T_{14}$ versus $T_{28}$ subsets, showing that some of these responses could be significantly decreased as a result of exposure to the contaminated sediments when compared to the reference test, most notoriously after 14 days of exposure. At $T_{28}$, all biomarker responses were more positively consistent. With respect to test $C_1$ *versus* $C_2$ subsets, MT1 only revealed some degree of consistency for the assays with sediment $C_1$, as AI for assays with sediment $C_2$. The HSP90α response did not reveal any consistency ($B_i$ = 0) for either case.

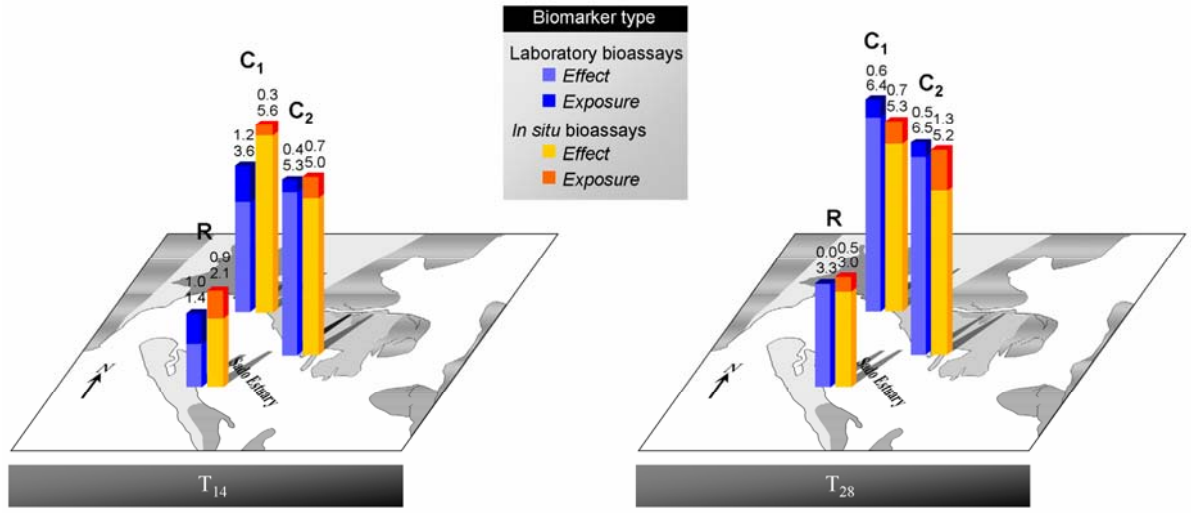


**Fig. 6.1.3.** Average variation coefficients (VCs) for biomarkers of effect (TSB, ENA and the histopathological indice $I_h$) and exposure (MT and CYP1A induction) from the simultaneous laboratory and field assays (spring) for each site: reference (R) and contaminated ($C_1$ and $C_2$). The VCs were estimated taking $T_0$ fish as the calibrator group in order to compare all three sites. The average VCs comprise both fall (laboratory) and spring (laboratory and *in situ*) bioassays.

*3.4. Integration of multi-level responses*

The proteomic screening detected nineteen deregulated cytosolic proteins in soles exposed to sediments through laboratory and *in situ* bioassays for 14 days (Costa et al., submitted, see Section 4.4). The matched proteins related to several functions, from anti-oxidative defence (e.g. 1-cysPrx) to gene transcription (TE2B) and energy production (Table 6.1.4). Correlation-based cluster analysis combining all measured responses from fish tested at spring collected at $T_{14}$ (laboratory and *in situ* assays) retrieved two major clusters (Fig. 6.1.6). The first cluster includes the VCs for all considered biomarkers of effect (TSB, ENA and $I_h$), the apoptotic indice and the VCs for the regulation of LDH and 1-cysPrx enzymes. The second cluster includes the remaining responses and can, on its turn, be subdivided in two other clusters. With the exception of CYP1A transcription, the VCs for the remaining genes were found in the same cluster, together with the regulation of two energy production-related enzymes (PGAM2 and Eno1), a lipid transporter (ApoA1), trypsinogen 1c (a trypsin protease precursor) and a hypothetic intracellular signalling protein, SPB1.

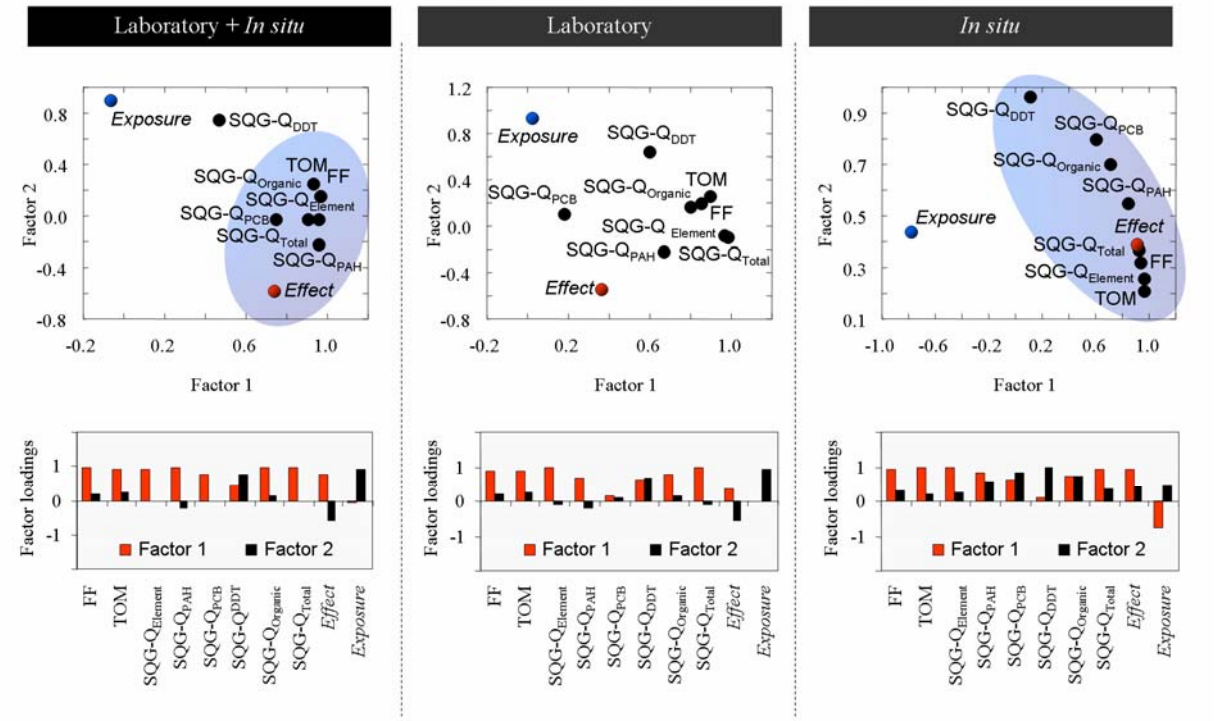


**Fig. 6.1.4.** Factorial analysis (extraction by principal components) by assay type (laboratory and *in situ*) comprising data from fall and spring assays integrating *SQG-Q*s as the measure of sediment risk and the average variation coefficients (VCs) for biomarkers of effect (TSB, ENA and the histopathological indice $I_h$) and exposure (MT and CYP1A induction), estimated considering $T_0$ animals as the calibrator group.

**Table 6.1.3.** Measured variation coefficients (VCs) of biomarker responses in soles exposed to the reference (R) and contaminated ($C_1$ and $C_2$) sediments relatively to $T_0$ fish (calibrator group, VC = 1). Boldface text indicates significant differences to exposure to the reference sediment at the same assay and sampling time (Mann-Whitney *U*, $p < 0.05$).

| | | | | Biomarker response variation coefficients | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Season** | **Assay type** | **Sampling time (days)** | **Site** | **TSB** | **ENA** | ***I*$_h$** | **CYP1A*** | **MT**** | **CAT** | **GPx** | **HSP90α** | **CASP3** | **AI** |
| Fall 2006 | | | | | | | | | | | | | |
| | Laboratory | 14 | R | *1.4 ± 0.6* | *1.2 ± 0.8* | *1.2 ± 0.6* | *2.01 ± 0.04* | *0.9 ± 0.4* | - | - | - | - | - |
| | | | $C_1$ | 1.9 ± 0.6 | 1.7 ± 1.4 | **2.1 ± 1.0** | **2.79 ± 0.06** | 1.0 ± 0.3 | - | - | - | - | - |
| | | | $C_2$ | **2.8 ± 1.2** | **4.3 ± 3.7** | **2.6 ± 1.3** | **0.88 ± 0.01** | 1.4 ± 0.5 | - | - | - | - | - |
| | | 28 | R | *1.6 ± 0.5* | *2.9 ± 1.9* | *1.5 ± 0.8* | *1.04 ± 0.03* | *1.5 ± 0.8* | - | - | - | - | - |
| | | | $C_1$ | **2.5 ± 0.7** | **5.2 ± 1.8** | **2.3 ± 0.8** | 1.08 ± 0.01 | **1.5 ± 0.6** | - | - | - | - | - |
| | | | $C_2$ | **3.8 ± 2.8** | **6.8 ± 3.6** | **3.4 ± 0.7** | **1.42 ± 0.02** | **2.5 ± 1.1** | - | - | - | - | - |
| Spring 2007 | | | | | | | | | | | | | |
| | Laboratory | 14 | R | *1.4 ± 0.7* | *1.7 ± 1.0* | *1.0 ± 0.8* | *1.06 ± 0.24* | *0.96 ± 0.02* | *0.46 ± 0.06* | *1.02 ± 0.08* | *1.70 ± 0.13* | *1.70 ± 0.25* | *1.9 ± 0.2* |
| | | | $C_1$ | **3.6 ± 0.5** | 3.4 ± 2.3 | **3.8 ± 1.4** | 0.83 ± 0.17 | **1.56 ± 0.09** | **0.65 ± 0.10** | **1.02 ± 0.07** | 2.50 ± 0.33 | **1.26 ± 0.16** | 1.5 ± 0.5 |
| | | | $C_2$ | **5.1 ± 1.0** | **6.7 ± 1.3** | **4.2 ± 2.4** | **0.04 ± 0.01** | **0.79 ± 0.04** | **0.02 ± 0.00** | **0.28 ± 0.09** | **0.17 ± 0.02** | **0.65 ± 0.07** | **2.9 ± 1.1** |
| | | 28 | R | *1.5 ± 0.6* | *3.8 ± 1.9* | *4.7 ± 1.5* | *0.02 ± 0.01* | *0.07 ± 0.01* | *0.01 ± 0.001* | *0.07 ± 0.03* | *0.06 ± 0.01* | *0.34 ± 0.04* | *3.6 ± 0.2* |
| | | | $C_1$ | **4.1 ± 0.4** | **7.3 ± 1.7** | **7.7 ± 2.1** | **0.18 ± 0.03** | **0.98 ± 0.17** | **0.02 ± 0.01** | **0.76 ± 0.10** | **0.39 ± 0.04** | **0.82 ± 0.16** | 3.1 ± 0.9 |
| | | | $C_2$ | **5.8 ± 1.8** | **9.2 ± 1.2** | 4.4 ± 2.0 | **0.35 ± 0.04** | **0.60 ± 0.13** | **0.28 ± 0.03** | **0.52 ± 0.03** | **0.39 ± 0.01** | **0.88 ± 0.11** | **5.3 ± 1.2** |
| | *In situ* | 14 | R | *2.5 ± 0.6* | *1.1 ± 0.8* | *2.8 ± 2.0* | *0.36 ± 0.06* | *1.40 ± 0.08* | *0.06 ± 0.01* | *1.21 ± 0.31* | *1.65 ± 0.20* | *0.71 ± 0.05* | *1.3 ± 0.7* |
| | | | $C_1$ | **5.4 ± 1.0** | 2.4 ± 1.6 | **8.9 ± 2.6** | **0.44 ± 0.05** | **0.21 ± 0.01** | **0.03 ± 0.01** | **0.53 ± 0.01** | 0.62 ± 0.05 | **0.40 ± 0.05** | **0.2 ± 0.2** |
| | | | $C_2$ | **4.2 ± 1.2** | **3.5 ± 1.6** | **7.2 ± 1.2** | **1.10 ± 0.10** | **0.21 ± 0.01** | **0.12 ± 0.02** | **0.84 ± 0.03** | **0.50 ± 0.03** | **0.71 ± 0.14** | **0.7 ± 0.3** |
| | | 28 | R | *4.6 ± 1.4* | *1.2 ± 0.9* | *3.2 ± 1.8* | *0.29 ± 0.09* | *0.68 ± 0.11* | *0.03 ± 0.02* | *1.73 ± 0.29* | *0.93 ± 0.07* | *1.18 ± 0.14* | *1.1 ± 0.7* |
| | | | $C_1$ | 4.8 ± 1.1 | **3.8 ± 1.1** | **6.9 ± 3.0** | **0.42 ± 0.08** | **1.05 ± 0.12** | **0.08 ± 0.002** | **0.48 ± 0.11** | 0.51 ± 0.02 | **1.99 ± 0.10** | 1.6 ± 0.2 |
| | | | $C_2$ | 3.4 ± 0.9 | **3.5 ± 2.3** | **8.6 ± 2.7** | **1.19 ± 0.07** | **1.39 ± 0.12** | 0.09 ± 0.05 | **2.55 ± 0.18** | **5.25 ± 0.51** | **1.99 ± 0.29** | 0.5 ± 0.4 |

*MT induction was determined by DPP-SMDE in the fall assays and by qRT-RT-PCR in the spring

**CYP induction was determined by ELISA in the fall assays and by qRT-RT-PCR in the spring

AI, hepatic apoptotic index; ENA; erythrocytic nuclear abnormalities; $I_h$, hepatic histopathological condition indice; TSB, total DNA strand breakage.

**Table 6.1.4.** Cytosolic proteins matched with Protein-Protein Blast (Altschul et al., 1997) and respective relative regulation factors after the 14 days of exposure to the reference (R) and contaminated sediments ($C_1$ and $C_2$). Regulation factors were estimated taking $T_0$ fish (calibrator group).

| | | | | | | | Average regulation factors ± standard deviation | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Protein information** | | | | | | | **Laboratory** | | | ***In situ*** | | |
| Function and designation | Abbreviation | SwissProt accession | Taxa[†] | Score | *e*–value | Matched peptides | R | $C_1$ | $C_2$ | R | $C_1$ | $C_2$ |
| *Aminoacid metabolism* | | | | | | | | | | | | |
| Fumarylacetoacetase | FAA | Q803S0 | 2,3 | 46 | $7\times10^{-6}$ | 1 | 1.9 ± 0.02 | 1.2 ± 0.03 | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.2 ± 0.1 | 1.9 ± 0.05 |
| Glutamine synthetase | Gln_Synt | Q4RVF3 | 1,2,3 | 72.4 | $2\times10^{-13}$ | 2 | 0.9 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.2 | 1.2 ± 0.02 | 1.0 ± 0.1 | 0.8 ± 0.1 |
| Glycine *N*-methyltransferase | GNMT | Q6P607 | 2,3 | 71 | $2\times10^{-13}$ | 4 | 1.4 ± 0.1 | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.03 | 1.4 ± 0.1 | 1.6 ± 0.1 |
| *Cell cycle progression* | | | | | | | | | | | | |
| MAPK activating protein PM28 | IST1 | P53990 | 2,3 | 76.3 | $2\times10^{-14}$ | 3 | 1.5 ± 0.1 | 1.5 ± 0.3 | 1.3 ± 0.2 | 2.0 ± 0.1 | 1.4 ± 0.1 | 2.1 ± 0.1 |
| *Cytoskeleton* | | | | | | | | | | | | |
| Beta Actin | Act | Q1HHC7 | 1,2,3 | 71 | $2\times10^{-15}$ | 8 | 1.4 ± 0.1 | 1.0 ± 0.1 | 1.2 ± 0.1 | 2.3 ± 0.2 | 1.4 ± 0.2 | 1.6 ± 0.1 |
| *Energy production* | | | | | | | | | | | | |
| Biphosphoglycerate mutase 2 | PGAM2 | B5XD74 | 2,3 | 52 | $1\times10^{-7}$ | 3 | 1.6 ± 0.1 | 1.1 ± 0.0 | 1.3 ± 0.3 | 1.0 ± 0.1 | 1.1 ± 0.2 | 0.9 ± 0.2 |
| Enolase 1 | Eno1 | C0H878 | 2,3 | 57.5 | $3\times10^{-9}$ | 4 | 1.4 ± 0.1 | 1.4 ± 0.2 | 1.1 ± 0.2 | 1.6 ± 0.2 | 1.5 ± 0.1 | 0.9 ± 0.1 |
| Enolase 3 | Eno3 | Q568G3 | 2,3 | 68.1 | $2\times10^{-12}$ | 1 | 1.7 ± 0.1 | 0.8 ± 0.2 | 0.7 ± 0.3 | 1.3 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.2 |
| Malate/*L*-lactate dehydrogenase | LDH | Q7T3D9 | 2,3 | 75.3 | $1\times10^{-14}$ | 2 | 1.3 ± 0.1 | 1.7 ± 0.2 | 1.3 ± 0.2 | 1.6 ± 0.2 | 1.8 ± 0.1 | 1.5 ± 0.2 |
| *Gene transcription* | | | | | | | | | | | | |
| Transcription initiation factor IIE subunit beta | T2EB | T2EB | 2,3 | 56.6 | $5\times10^{-9}$ | 2 | 3.0 ± 0.1 | 1.3 ± 0.1 | 1.4 ± 0.2 | 2.3 ± 0.1 | 2.7 ± 0.2 | 2.1 ± 0.1 |
| *Intracellular signalling* | | | | | | | | | | | | |
| Selenium binding protein 1 | SBP1 | Q6PHD9 | 2,3 | 69.7 | $1\times10^{-12}$ | 3 | 2.7 ± 0.1 | 2.0 ± 0.2 | 2.2 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.0 | 1.3 ± 0.3 |
| *Lipid transport* | | | | | | | | | | | | |
| Apolipoprotein A-1 | ApoA1 | O42363 | 1,2,3 | 60 | $4\times10^{-10}$ | 3 | 1.4 ± 0.1 | 1.4 ± 0.2 | 1.1 ± 0.2 | 1.6 ± 0.2 | 1.5 ± 0.1 | 0.9 ± 0.1 |
| *Oxidoreductase* | | | | | | | | | | | | |
| 17-beta hydroxysteroid dehydrogenase | HSD | Q7T2J0 | 1,2,3 | 83.3 | $4\times10^{-17}$ | 4 | 2.0 ± 0.1 | 1.4 ± 0.2 | 1.4 ± 0.1 | 2.4 ± 0.1 | 2.6 ± 0.1 | 1.8 ± 0.1 |
| 1-cys peroxiredoxin | 1-cysPrx | Q8AWH6 | 1,2,3 | 41.8 | $1\times10^{-6}$ | 2 | 0.7 ± 0.1 | 1.3 ± 0.3 | 1.5 ± 0.2 | 0.9 ± 0.2 | 1.2 ± 0.1 | 0.8 ± 0.2 |
| Aldehyde dehydrogenase family | Aldh | Q98TM9 | 1,2,3 | 63 | $6\times10^{-13}$ | 5 | 2.0 ± 0.05 | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.6 ± 0.2 | 1.6 ± 0.2 | 2.1 ± 0.1 |
| *Proteolysis* | | | | | | | | | | | | |
| Cathepsin Z | CatZ | Q58HF4 | 1,2,3 | 77 | $8\times10^{-15}$ | 1 | 1.2 ± 0.05 | 1.0 ± 0.2 | 1.0 ± 0.6 | 1.7 ± 0.3 | 1.4 ± 0.2 | 2.0 ± 0.1 |
| Trypsinogen 1c | TRP1c | A7VMR6 | 1,2,3 | 54.5 | $2\times10^{-10}$ | 2 | 0.9 ± 0.1 | 3.8 ± 0.1 | 1.7 ± 0.05 | 0.5 ± 0.1 | 0.2 ± 0.4 | *n.o.* |
| *Proteolysis (ATP dependent)* | | | | | | | | | | | | |
| Proteasome subunit beta type 2 | PSB2 | Q6DHI9 | 2,3 | 52.4 | $9\times10^{-8}$ | 2 | 2.4 ± 0.1 | 1.4 ± 0.1 | 1.2 ± 0.2 | 2.3 ± 0.1 | 2.2 ± 0.2 | 2.4 ± 0.1 |
| Proteasome subunit beta type 9 | PSB9 | Q9DD33 | 1,2,3 | 94.9 | $6\times10^{-20}$ | 2 | 1.3 ± 0.1 | 0.8 ± 0.2 | 0.9 ± 0.3 | 1.6 ± 0.1 | 1.6 ± 0.1 | 1.1 ± 0.1 |

[†]All Non-Redundant Database (NCBI, USA) taxa from which peptide matched was obtained: 1-Order Pleuronectiformes; 2-Class Actinopterygii; 3-Phylum Chordata. *n.o.* - no expression observed in any of the triplicate gels obtained per experimental condition.

## 4. Discussion

The present study has shown that, at least with respect to exposure to estuarine sediments contaminated by mixed classes of substances, there is a clear distinction between biomarkers (or potential biomarkers) that reflect lesions directly than those that should represent some sort of response or defence mechanism to xenobiotic insult. In addition, it has also been demonstrated that the bioassay approach can yield different results when performed under controlled or natural conditions. However, these were not the only factors contributing to variation of effects and responses to mixed environmental contamination: other external variables, such as seasonal changes in sediment characteristics (especially contaminant load) and unknown noise variables can greatly influence the results. With respect to the organisms' responses *per se* (internal variables), it can be inferred that stress affects multiple biological processes producing an interlinked pattern that returns mandatory the need to interpret the results from biological analysis with caution. This combination of factors produced a series of unexpected results, such as highest genotoxicity in whole-blood and highest hepatic histopathological lesions in fish exposed to sediments from site $C_2$ (contaminated by organic substances, especially PAHs) in the fall assays when, in fact, it was sediment $C_1$ (contaminated by both metallic and organic substances) the one globally most contaminated, a pattern that (not shown in the present paper) was observed for other organs, namely gills (Costa et al., 2009b) and kidney, where severe proliferative disease (PKD) was observed (Costa et al., 2010a). Bioaccumulation, on its turn, (not shown here) which was determined for inorganic and organic contaminants in fish tested in the fall, was very variable, overall very low and little linked to MT and CYP1A induction, presumably by the relatively low levels of sediment metals and the ability of fish to activate and eliminate PAHs (see Costa et al., 2009a, for specifics). Still, in spite of the aforementioned constraints, to which can be added the fact that the contaminated sediments under scope can be considered moderately contaminated according to the criteria proposed by MacDonald et al. (2004), the integrative biomarker approach hereby present was purposeful in the distinction between sediments regarded as uncontaminated from those suspected to be of risk.

It has also been demonstrated that, under the specific circumstances of assessment, establishing accurate cause-effect relationships between specific contaminants concentrations and toxicity is complicated. However, the employment of multi-level biomarker, or potential biomarker, responses (that in essence relate to different levels of biological organization) can be effective in the distinction between contaminated and uncontaminated sediments, identify more suitable biomarkers to complex mixtures and contribute to the understanding of the mechanisms of response to xenobiotic induced stress and at some extent segregate the effects induced by organic and inorganic substances. The results need, therefore, to be debated under the scopes of the methodological approach, sediment geochemistry and the biology of toxicity.

*4.1. Laboratory* versus *field bioassays*

As a consequence of the increased bioavailability, the contaminants may have their individual potential toxicity amplified, as well as the possible interaction effects between the substances (see below). Although the usefulness and adequacy of both types of bioassays in monitoring studies has been demonstrated (e.g. Riba et al., 2005), few papers deal directly with the comparison between laboratory and field bioassays. However, differences between the two approaches, with respect to biological responses in benthic fish, have already been reported as well as the resulting difficulties in assessing experimental noise from true toxicopathic results (see, e.g., Vethaak et al., 1996; Hatch and Burton, 1999). It has been earlier hypothesized that the combination of low Eh with high FF and TOM contents might have in fact favoured the release of contaminants from the sediments during the laboratory assays as a result of disturbance from collection, handling and also animal-driven resuspension (e.g. Costa et al., 2008b, 2009a, 2010c). In fact, it has been reported that the combination between disturbance and the anoxic-oxic shift occurring when sediments are exposed to oxygenated water (as occurred during the laboratory experiment) favoured the release of substances trapped by organic matter and fine particles back to the water column, thus rendering them bioavailable (see Eggleton and Thomas, 2004). Furthermore, resuspended contaminants tend to persist for an extended time in the water column as a consequence of turbation (Caetano et al., 2003; Atkinson, 2007).

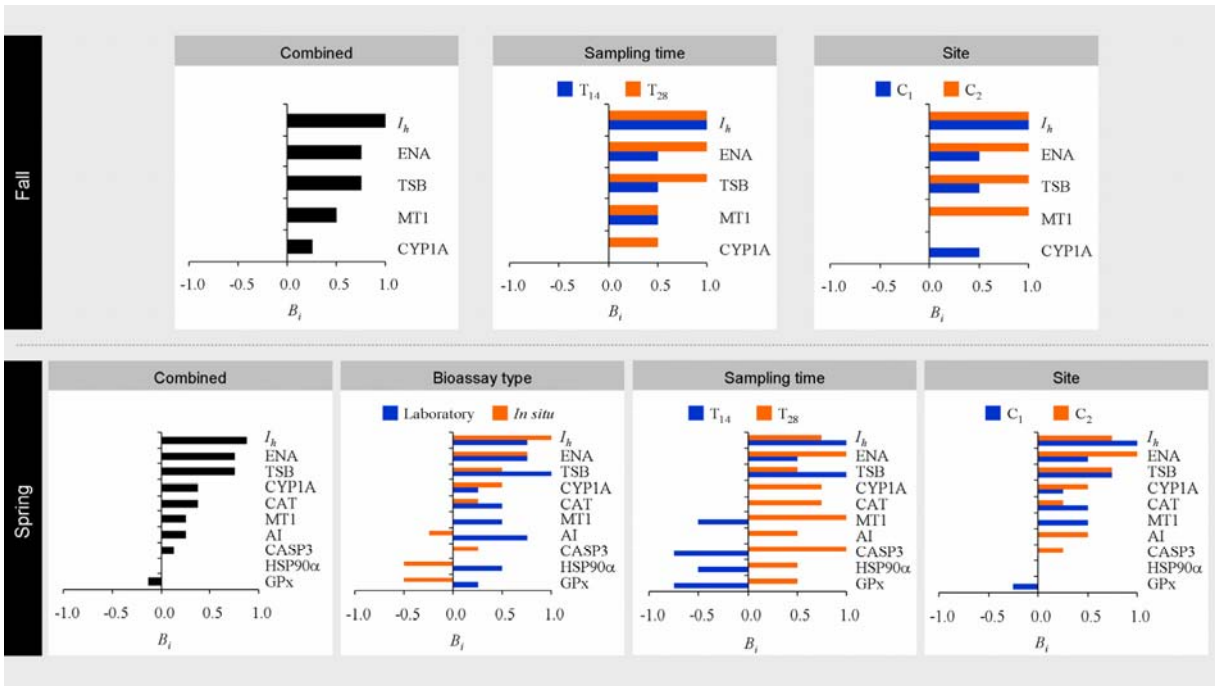


**Fig. 6.1.5.** Consistency of biomarker responses per assay type and season. The biomarker consistency indice ($B_i$, see formula [3]) ranges between -1 (consistent decrease between exposure to the contaminated and the reference sediments per experimental subset) and 1 (consistent increase).

Field bioassays, on their turn, can be affected by unaccounted background variables that are difficult to manage. At the spring assays, fish collected at $T_{28}$, caged at the reference site, revealed unexpectedly higher concentration of lipid peroxides in blood plasma than animals from the

contaminated sites with a parallel increase in whole-blood TSB levels but not ENA (Costa et al., 2010c). Gene expression analysis showed that GPx transcription was also elevated in these animals (Costa et al., submitted, Section 4.3). This was likely due to increased oxidative stress caused by poor access to food items since *in situ* R-tested fish for 28 days did not have in their digestive tracts the small gastropods and bivalves that were commonly found in soles exposed at other sites, being starvation a known cause of increased lipid peroxidation due to disrupted lipid metabolism in fish (Morales et al., 2004; Pascual et al., 2003). It can be suspected that food deprivation occurred due to the higher hydrodynamics of the site: a partial removal of the sediments was observed when retrieving the cages, therefore limiting the animals' access to preys. Nevertheless, histopathological condition of the livers of the soles did not appear to have been significantly affected by the occurrence as to impair a comparison between the reference test and the tests in the contaminated sites, the similar occurring with the other transcriptomic responses.

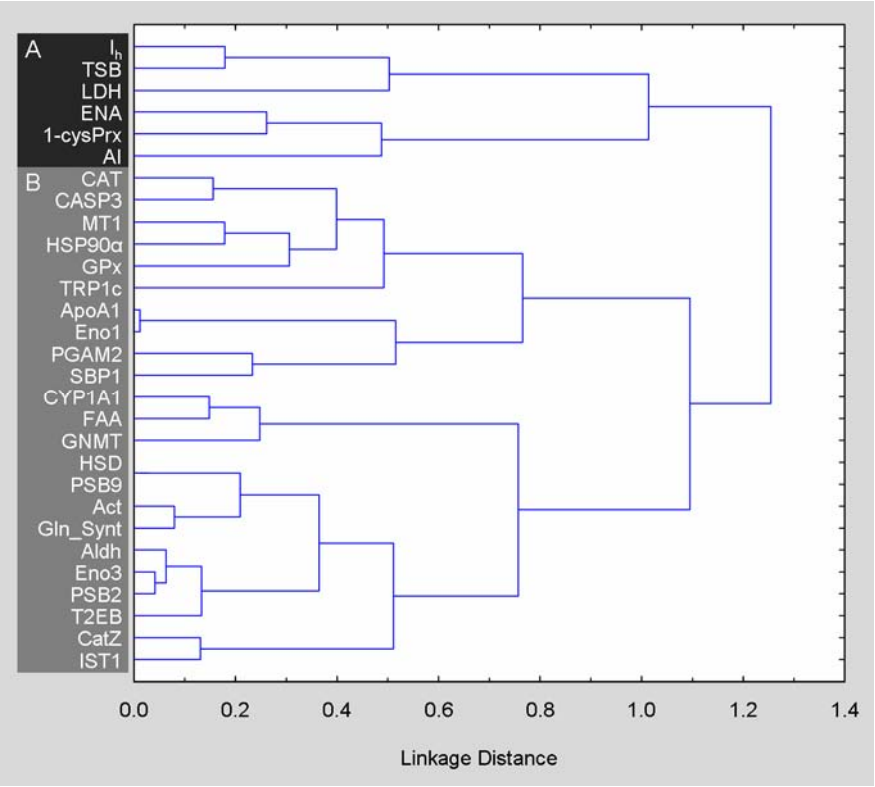


**Fig. 6.1.6.** Correlation-based cluster analysis (1-Pearson *r* statistic was used as distance metric) combining all hepatic biomarker responses (given as variation coefficients to $T_0$ fish) from field- and laboratory-tested fish (April assays) after 14 days of exposure to any surveyed sediment. Unweighted pair-group average was employed as amalgamation rule. Two major clusters are discernible, A) comprising all considered biomarkers of effects plus the apoptotic indice AI and an oxidoreductase enzyme, 1-*cys* Peroxiredoxin (1-cysPrx), and the lactate dehydrogenase (LDH) energy production-related enzyme and B), which includes all other responses measured by "omic" approaches and that relate to multiple cellular functions, including transcription of MT1, CAT, CYP1A1 and other genes plus several proteins of distinct functions, from proteolysis (e.g. TRP1c and CatZ) to energy production (such as Eno1 and Eno3).

*4.2. Sediment geochemistry and contamination profiles*

Some authors argued that one of the reasons why "classical" biomarkers responses may yield unexpected responses deriving from exposure to low concentrations of a specific set of "inducers" within a toxic mixture of substances. It is the case, for instance, of reduced or non-detected MT overexpression in aquatic animals exposed to mixtures containing low levels of Cd or other strong MT inducers (Costa et al., 2008a; Mouneyrac et al., 2002). It can therefore be assumed that moderate levels of contamination pose yet another constraint to biomarker interpretation even when adverse effects to organisms are detected as in the present study. Furthermore, natural seasonal changes in sediment geochemistry need to be taken into consideration when monitoring dynamic environments such as estuaries. Although the overall levels of contamination of the prospected sites was found to have the same magnitude as those observed from previous recent studies, shifts were observed from fall to spring assays, such the increase of metal concentrations in station $C_2$, in which was observed to have a similar level of contamination than the reference station in the fall. This increase in metal concentrations in the spring led to the somewhat unexpected similarity between *SQG-Q*s for sites $C_1$ and $C_2$, regardless of the inclusion of Hg in the chemical analysis to the sediments. Still, the levels of contamination by this metal for the Sado estuary have been found to be low to moderate (Lillebø et al., 2010). Other fluctuations were observed for organic contaminants, namely a small increase in total PAHs from fall to spring, a tendency that is opposite to what was inferred from previous research (Neuparth et al., 2005). Regarding organochlorines, PCB levels were observed to be relatively low and always lower than the *TEL* guideline, with little variation being found between seasons. For DDTs, however, a significant seasonal variation has been found in site $C_1$, the most contaminated by *pp*'DDT in the fall (above the *TEL* guideline, indicating potential risk) but showing a considerable increase in the spring, loosing its rank to sediment $C_2$, where no noticeable seasonal variation was observed. The levels of DDTs for this station were consistent with the values found for the lower estuary about a decade ago (Gil and Vale, 1999). These temporal fluctuations of contaminants in the area are in accordance with previous studies (Cortesão and Vale, 1996), reinforcing the premise that seasonality can in fact cause variation in contamination profiles, resulting in inter-seasonal variation in biomarker responses and overall sediment risk assessment, as observed from the fall to the spring bioassays.

*4.3. The effects of contaminant interactions on biomarker responses*

Interaction effects, as previously proposed by the authors (e.g. Costa et al., 2009b) are likely scenarios when assessing toxicity in animals exposed to complicated mixtures of contaminants. Such interactions include agonist effects (meaning that the response is the sum of the individual responses triggered by the contaminants at stake), synergistic (when co-exposure causes a response not achievable by any agent on it won) and antagonist (inhibitory) effects. Amongst these cases, antagonist effects between contaminants have already been identified and reported by several authors.

It is the case, for instance, of co-exposure to metals and metalloids inhibiting CYP upregulation and activity by strong inducers such as PAHs (Vakharia et al., 2001; Spink et al., 2002; Sorrentino et al., 2005). Co-exposure to metals and PAHs (e.g. Cd and benzo[a]pyrene, respectively) has also reported to inhibit apoptosis and even the expression of glutathione peroxidase (Costa et al., 2010b). Accordingly, these biomarkers at some point yielded unexpected lower values in exposed to contaminated sediments in the spring (especially $C_2$, the most contaminated by PAHs but also contaminated by some metals), most notoriously at $T_{14}$.

It has been hypothesized in previous works that co-exposure to metals and PAHs may, in part, explain why adverse effects (i.e. DNA damage and histopathological alterations) were higher in $C_2$--tested fish and not $C_1$ (the most contaminated sediment), especially at $T_{14}$ and in the fall assays, where a greater difference between the contamination levels of the two sediments was more evident. PAHs are liposoluble compounds, detoxified through activation by CYP1A monooxygenase enzymes, which renders them more hydrophilic, thus more easily excretable. However, not only activated PAHs, such as diol-epoxides and quinones are highly genotoxic but the activation process itself generates reactive oxygen species (ROS) as by-products (e.g. Lemaire and Livingstone, 1997). Other response mechanisms, such as apoptosis are known to be triggered by oxidative stress. Therefore, impairment of PAH activation can, at a short term, avoid oxidative damage and delay downstream responses to injury such as apoptosis (also termed programmed cell death or PCD) since apoptosis consists of a cytological process that intends to “dismantle” heavily-damaged cells to avoid inflammation and scattering of toxic cellular debris resulting from necrosis and the propagation of mutations. The caspase 3 (CASP3) enzyme a key effector caspase in the apoptotic process and is though to have its regulation modulated by oxidative stress (see Wyllie, 2010, for a recent review). Accordingly, CASP3 transcription, apoptosis, DNA and histological damage were observed to be correlated in fish exposed for 14 days in the spring assays (Fig. 6.1.6). However, the allocation of CYP1A in a different cluster may indicate that other factors rather than CYP1A expression (and activity) are impaired by contaminant interaction. The inclusion of an anti-oxidative stress enzyme (a peroxiredoxin) in the same cluster can also indicate that ROS are a chief factor in the balance between the effects and responses to toxicity. In fact, peroxiredoxins are though to be effective anti-oxidant enzymes (Chen et al., 2000), however little surveyed in toxicological studies. Interestingly, an enzyme involved in anaerobic respiration, lactate dehydrogenase (LDH) was also included in this same cluster, which could indicate impairment of oxidative metabolism and aerobic respiration. As an example, LDH activity has been found to occur as a consequence of exposure to styrene in CYP1 deficient mice, therefore with impaired catabolism of this substance (Carlson, 2010).

Regarding another “classical” biomarker, MT induction, there are many contradictory studies whose combined interpretation confirms the complexity of exposure to contaminant combinations: while a synergistic effect was detected when injecting sea basses with both Cu (a known MT inducer, well represent in the surveyed sediments $C_1$ and $C_2$ at both seasons) and B[a]P (with levels above the *PEL SQG* being found in $C_1$ and $C_2$ sediments in the spring) when the same independent dosages of

both caused a reduction in the protein regulation (Roméo et al., 1997). Conversely, MT induction was found to be suppressed by the PAH methylcholanthrene in trout hepatocyte cultures exposed to a combination of metals (Risso-de Faverney et al., 2000). Although these might be just a few examples, biomarker responses were most likely affected by these and other contaminant interaction effects, which should thus be regarded as a fundamental constraint when surveying the toxicity of intricate mixtures of xenobiotics, an issue that still needs much research.

*4.4. Biomarker responses are affected by general metabolic disruption*

Most "classical" biomarkers that relate to gene expression (typically surveyed at the transcript and final protein levels) are considered to respond positively (i.e. to have their responses increased) to exposure, such as CYP1A or MT induction for aromatic hydrocarbons and metals, respectively. The variation along the assays' duration indicates that contaminant interaction should not be the sole accountable affects for unexpected observations, especially those related to the differences between $T_{14}$- and $T_{28}$-sampled fish. The increase in non-lesion biomarker responses (hereby referred to as biomarkers of "exposure" for simplification purposes) could more consistently distinguish animals exposed to contaminated from uncontaminated sediments after 28 days exposure, for all bioassays (Fig. 6.1.5). In fact, a very significant downregulation of most transcription responses was observed in the spring assays, at $T_{14}$, some exhibiting the very opposite response at $T_{28}$ (as for GPx and CASP3). The downregulation of responses is not, however, positively linked to hepatic lesions. Also, the increase in $I_h$ was observed to significantly distinguish exposure to contaminated *versus* uncontaminated sediments for all tests, at $T_{14}$, revealing very significant liver damage occurring at the assays midterm. At $T_{28}$, however, such lesions lost some of their significance in this distinction, with fish exposed *in situ* to sediment $C_2$ actually revealing some sort of amelioration of hepatic damage at $T_{28}$ (Table 6.1.3).

Although there is surprisingly little literature regarding the integration of biomarker responses in toxicological stress models some authors have already debated the importance of understanding the stress curves in the interpretation of biomarkers endpoints (Triebskorn et al., 1997). Even considering the differences between all bioassays performed, the animals sampled at $T_{14}$ (the assays' midterm), were mostly likely still enduring the homeostatic and metabolic disturbance triggered by exposure, while fish collected at the end of the assays exhibited ability to respond and perhaps attenuate some of the negative effects (see Steinberg et al., 2008). The proteomic screening performed on $T_{14}$-collected fish (from the spring assays) is in accordance with this statement since, especially in laboratory--exposed fish to sediments $C_1$ and $C_2$, most matched proteins were downregulated compared to R--tested or even $T_0$ animals (refer to Table 6.1.4 and Section 4.4 for details).

In general terms, the 28-day bioassays reveal the existence of two distinct steps that fit the first two phases of the acknowledged stress curve model (Fig. 6.1.7). The reduced or impaired responses observed at $T_{14}$ displayed by fish exposed to the contaminated sediments compared to those from the

reference test likely reflect an initial disturbance phase that derives from exposure and in part to the inevitable change in the animals' environment when they were allocated in the testing apparatus. The second step, therefore, reflects the beginning of the response, or recovery, phase when the fish regained balance of biological functions, as inferred from the results obtained at $T_{28}$. These results provide yet another evidence that the time of exposure is a decisive factor when interpreting biomarker data and indicated that null or even lowered responses does not mean absence of pressure onto organisms. The results indicate that metabolic disruption affects several baseline biological mechanisms, from energy production to gene transcription itself.

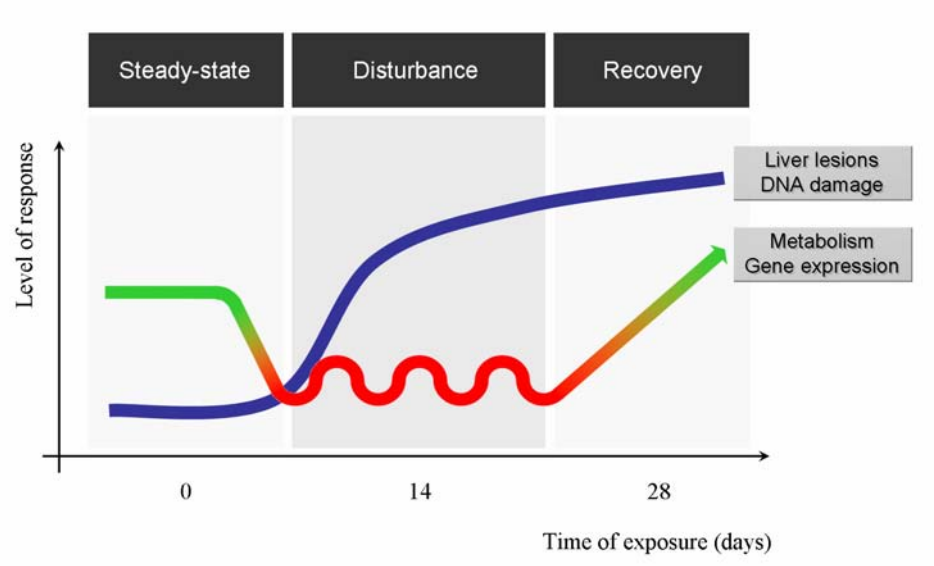


**Fig. 6.1.7.** A diagram of the partial contaminant-induced stress phase model juxtaposed with the assay's duration (28 days) and sampling times (at days 0, 14 and 28).

*4.5. Global biomarker evaluation*

The combination of moderate levels of contamination with the existence of a complex mixture of multiple classes of contaminants considerably diluted the variation between contaminated and reference (clean) sediments. Still, biomarkers that reflect some sort of damage were more consistent to detect differences between contaminated and uncontaminated sediments than protein induction-related ones. The differences between the surveyed contaminated sediments, nevertheless, could not be clearly detected as to link to the actual levels of contaminants and their potential risk to organisms. This is likely due to the loss of specificity of the measured responses in face of the detected levels of contaminant mixtures, to the interaction effects between contaminants and to the temporal variation of sediment pollutants, since a considerable increase in overall contamination in one of the surveyed sediments from fall to spring led to a similar potential of risk between contaminated sites.

In spite of the characteristics of the study area and the observed constraints in assessing toxicity of sediment-bound xenobiotics, it was observed that moderately contaminated sediments can elicit adverse effects to benthic fish and that surveying multi-level lesions (namely DNA damage and

multiple histopathological changes) can provide a more clear-cut distinction between clean and contaminated sites when complex mixtures of pollutants are involved, regardless of assay type. The "omic" surveys (transcriptomic and proteomic), on the other hand, permitted a screening of multiple responses, some of which revealed potential for predictive environmental toxicology, such as CASP3 and peroxiredoxin expression, which clearly deserve further research. These surveys, by integration of these multiple responses gave important insight on the mechanistics of exposure to mixtures of contaminants, namely the central role of oxidative stress, energy production imbalance and proteolysis. Furthermore, it is clear that different testing conditions, time of exposure and contaminant levels can provoke very different expression patterns, even in genes involved in similar processes, as CAT and GPx in anti-oxidative defence. Surveying multiple responses can thus aid filling in the gaps when a particular trait fails to produce significant outcomes or reveals results that, at a glance, contradict those predicted by theory.

Both laboratory and field bioassays could effectively segregate exposure to reference and contaminated sediments but the biomarkers approach revealed that the effects and responses to toxicity imposed by the two approaches can be very distinct, with histopathological alterations being one of the most consistent biomarker sets. Laboratory studies, however cheaper and more expedite, may globally tend to overestimate toxicity, especially by favouring the release of contaminants trapped in the sediments upon handling and subsequent re-oxygenation. On the other hand, they are not affected by unaccountable environmental variables that introduce experimental noise in the field. Although sediment disturbance events by natural causes such as storms and heavy river runoffs or anthropogenic modifications such as dredgings are common events in estuaries, laboratory studies may thus produce results that are less realistic in ecological terms in steady-state environments, which may render this approach somewhat less effective for practical monitoring programmes. Nevertheless, studies in controlled environments remain very valuable for more mechanistic surveys. The choice of the adequate bioassay methodology is, therefore, not an easy one and requires a careful balance between cost, logistics, fundamental objectives and the need to reduce experimental noise with the least prejudice of ecological relevance.

### *4.6. Concluding remarks*

The combination of moderate levels of contamination with the existence of a complex mixture of multiple classes of contaminants considerably diluted the variation between contaminated and reference (clean) sediments. Still, biomarkers that reflect some sort of damage were more consistent to detect differences between contaminated and uncontaminated sediments than protein induction-related ones. The differences between the surveyed contaminated sediments, nevertheless, could not be clearly detected as to link to the actual levels of contaminants and their potential risk to organisms. This is likely due to the loss of specificity of the measured responses in face of the detected levels of contaminant mixtures, to the interaction effects between contaminants and to the temporal variation of

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In spite of the characteristics of the study area and the observed constraints in assessing toxicity of sediment-bound xenobiotics, it was observed that moderately contaminated sediments can elicit adverse effects to benthic fish and that surveying lesions can provide a more clear-cut distinction between clean and contaminated sites when complex mixtures of pollutants are involved. The “omic” surveys (transcriptomic and proteomic), on the other hand, permitted a screening of multiple responses, some of which revealed potential for predictive environmental toxicology, such as CASP3 and peroxiredoxin expression, which clearly deserve further research. These surveys, by integration of these multiple responses gave important insight on the mechanistics of exposure to mixtures of contaminants, namely the central role of oxidative stress, energy production imbalance and proteolysis. Furthermore, it is clear that different testing conditions, time of exposure and contaminant levels can provoke very different expression patterns, even in genes involved in similar processes, as CAT and GPx in anti-oxidative defence. Surveying multiple responses can thus aid filling in the gaps when a particular trait fails to produce significant outcomes or reveals results that, at a glance, contradict those predicted by theory.

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## Acknowledgements

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB/57281/104) and financed by FEDER (European Fund for Regional Development). P.M.

Costa is supported by an FCT PhD grant (SFRH/BD/28465/2006). The authors wish to thank E. Chicano-Gálvez, J. Alhama and J. Lopez Barea, (Universidad de Córdoba), C. Miguel (ITQB), M. Diniz (REQUIMTE) and T.S. Neuparth (CIIMAR) for their support. The authors are also thankful to APSS (Administração dos Portos de Setúbal e Sesimbra, SA), RNES (Reserva Natural do Estuário do Sado) and M. Caetano, M. Martins, A. Ferreira, P. Pousão, V. Branco, R. Cesário and J. Raimundo (IPIMAR).

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## 6.2. Concluding remarks and future perspectives

The present work demonstrated that a biomarker survey in a benthic fish exposed to a complex contaminant matrix, as a natural estuarine sediment, may yield results that at a first glimpse appear either difficult to interpret or even inconsistent with published literature. These results derive from the intricate nature of the sediments themselves and from the biological alterations that are triggered by exposure. The results also show that some technical aspects may be very important when interpreting bioassay-derived data, especially those that relate to the differences between laboratory and field (*in situ*) testing. At a secondary stage, however, the present work showed that constraints of assessing sediment toxicity can be bypassed through the integration of multi-level biological responses with sediment contamination profiles. Such an outcome has been achieved by a tiered approach to toxicity testing, taking the liver as the main (but not exclusive) target organ due to its role in contaminant accumulation, transformation and detoxification. At a first stage, fundamental bioassay and biomarker approaches were technically developed and evaluated (Chapter 2). The following step (Chapter 3) involved sediment testing in the laboratory focusing at three levels of biomarker responses: genotoxicity, biochemical and histological. This stage allowed the identification of constraints and difficulties resulting from fish exposure to complex mixtures of sediment-bound toxicants, as well as provided first-stage insights on the biological mechanisms underlying contaminant interactions. The information hereby developed was further improved by the following stage, which comprised simultaneous laboratory and *in situ* bioassays (Chapter 4), which added a new level of biomarkers (or potential biomarkers) to the aforesaid deriving from "omic" approaches, namely toxicogenomics and proteomics, that provided much needed new information, from gene sequencing to novel target protein identification. This step further unravelled the effects of contaminant interactions and the relationship between the sediments' levels of contamination and the alterations imposed to fish, from molecular to histological levels. Furthermore, it was now possible to compare the assets and constraints between the two distinct bioassay methodologies: laboratorial and *in situ*, an important achievement accomplished by the present work since such methodological comparison is virtually absent from available literature. Due to the complexity of surveying the biological mechanisms of contaminant co-exposure, a fully laboratorial bioassay procedure was undertaken, taking cadmium and the PAH benzo[a]pyrene as model xenobiotics (Chapter 5). This experiment combined proteomic with histological and cytological analyses and again provided much novel information on the intricate chain of biological mechanisms triggered exposure to combined toxicants and aided answering many questions pending from the previous bioassays. Finally, the outcomes from all sediment bioassays were integrated in order to provide a wide picture on mechanistic aspects of sediment-bound toxicity and to offer a global evaluation of the surveyed biomarkers with especial respect to their adequacy in predicting adverse effects to organisms (Chapter 6). Both aspects constitute, in result and approach, yet another novelty achieved by the present work.

Among the many constraints of testing natural sediments, the existence of multiple classes of organic and inorganic xenobiotics is one of the major contributors since, at the biological level; there are interaction effects that can effectively mask biomarker measurements. It is the case, for instance, of the known antagonist interaction between metals and polycyclic aromatic hydrocarbons (PAHs) towards CYP1A (cytochrome P450) activity and induction, apoptosis and many others, besides the probable existence of agonist and synergistic effects evidenced by "omics" combined with histological and cytological analyses (see Section 5.1), which is in good accordance with the results from the sediment laboratory assays (refer to Section 4.3). In addition, moderate levels of multiple contaminants can greatly dilute the generally recognized specificity of some biomarkers such as metallothionein (MT) or CYP for metals and aromatic hydrocarbons, respectively, by failing to trigger a clear-cut response (see Section 3.2). It seems, therefore, that no simple answer can disclose the full potential of sediment-bound contamination (or the intrinsic biological mechanisms by which it becomes toxic to organisms) in presence of multiple classes of toxicants.

As an indicator (but not an effective biomarker) of exposure to chemicals, bioaccumulation, on its turn, was not observed to be a very elucidative outcome from the assays were it was surveyed. Although generally regarded, at best, as an indicator and not an effective biomarker, it still provides useful information to be integrated within the pool of analysed biomarkers. Bioaccumulation revealed that 28-day bioassays may not be long enough to produce a clear response and also that it is dependent of the organisms' own mechanisms of storage and excretion of xenobiotics as well as it is highly reliant on the factors affecting bioavailability and substance speciation (Section 3.2).

In spite of all the difficulties in assessing sediment toxicity, the multi-biomarker approach was able to make a distinction between exposure to contaminated sediments from exposure to reference ("clean") ones; even though the consistency of biomarker responses was notably higher for those reflecting some degree of damage, with overall little or no specificity to a given class of xenobiotics. It can thus be inferred that at least such biomarkers can be successfully employed as an effective toxicity line-of--evidence for an ERA strategy in the study area, as is the case of histological lesions and DNA damage. It is, therefore, clear that even low to moderate levels of contamination can cause adverse effects to organisms that are reflected in several degrees of chronic disease affecting multiple organs and functions: from hepatic diffuse forms of pre-neoplasia and necrosis to proliferative kidney disease and gill epithelia alterations that may lead, at least, to impairment of osmotic balance by affecting chloride cells.

It must at this point be noted, however, that biomarkers that indicate lesions do not provide, on their own, much insight on the mechanisms of multi-contaminant toxicity and may still be prone to be modulated, for instance, by contaminant interactions, xenobiotic bioavailability and by the testing procedures *per se*. Instead, they provide an assessment of the health status of the tissue or organ (and

subsequently the individual's). It was the integration of multiple biomarkers of "exposure", i.e., those that reflect the metabolic changes and the responses set off by exposure that provided much insight on the complex biological framework that underlies xenobiotic-induced stress. In the present study, such biomarkers (or potential biomarkers) consisted of gene expression-related responses in the liver, either by surveying transcription of changes to protein content. It has been shown that metabolic alterations are much more complex than just the induction of a given protein in face of stress (as MT, CYP or anti-oxidant enzymes like catalase and glutathione peroxidase). Actually, the metabolic changes are reflected in energy budget, cell structure, cell cycle progression and many other pathways that do not function isolated but rather form an intricate web. As an example, gene expression is ATP dependent, therefore linked to energy production which, on its turn, is modulated by oxidative status. Also, when injured, cells might undergo "dismantling" by apoptosis or undergo less controlled cell death by other processes (as autophagy and necrosis) that involve proteolytic enzymes, both ATP-dependent (as the proteasome) or independent (as cathepsins) which will patently constitute a confounding factor if the toxicologist is measuring a response that is based on the content of a certain protein.

Advanced screening techniques for responses to contamination like the "omics" approaches confirmed that the mechanisms by which mixtures of contaminants cause adverse effects to organisms and elicit responses are diverse and complex. The molecular tools clearly showed that toxicity occurs not just as a direct effect of a given substance but also, and perhaps more importantly, because a substance (or a mixture) impairs responses that should prevent or ameliorate damage. As so, toxic effects may result from reduced ability to respond to contamination. Again, it is clear that one might be misled to believe that if a certain response has not changed or even resulted in a much lower outcome than theoretically expected for a specific contaminant, then this substance is not present as a "pollutant", i.e., in toxic concentrations. This means that there is hardly a "pure" biomarker response when an organism is exposed to a complex setting of xenobiotics. It has to be kept in mind, though, that there are many other pathways that were not surveyed in the present work but that should constitute highly relevant subjects for the future, such as the effects of exposure on the DNA repair machinery, apoptosis control or even gene transcription regulation. Such issues, although considered of high importance by other toxicological surveys (especially pharmaceutical and biomedical), are little or not at all addressed regarding environmental toxicants. In addition, it would be of importance in future studies to further develop screening "omic" techniques with this species to address the effects and responses of sediment-bound contaminants such as proteomics on membrane and microsomal proteins and wide--range state-of-the-art transcriptomics such as DNA microarray chips, to which the present work positively contributed by adding sequences of toxicologically relevant genes to open-access databases.

It is thus likely that, regarding the problem of testing complicated contaminant mixtures, instead of looking for a specific response or effect, a researcher should strive to find patterns from multiple data. The main advantages derive essentially from three aspects: i) by surveying multiple responses one

biomarker failing to produce consistent results to distinguish contaminated from uncontaminated sediments can perhaps be bypassed by another similar response; ii) by integrating these multiple (and preferably multi-level) responses and effects, important contributions can be given to the basal biological mechanisms underlying chemical insult and iii) combining multiple responses with sediment contamination profiles gives a more realistic and complete evaluation of the sediments' potential to cause adverse effects to organisms.

It must be reinforced that the biological information produced would be of limited use without a proper characterization of the tested sediments. It was this information that allowed establishing some degree of cause-effect relationships even when contaminant interactions were likely at stake. Under this point of view, the assays with juvenile *Solea senegalensis* successfully provided two major components of a Sediment Quality Triad perspective to monitor the ecological risk of the estuary's sediment contamination: toxicity (effects to organisms) and sediment geochemistry. Yet missing and out of the scope of the present work, remains the study of changes in the ecological structure of the surveyed sites, for instance through the assessment of changes in the macrofaunal composition, an issue that should be addressed in the future. Considering its capability to respond, its benthic nature and its availability from mariculture facilities, this species proves to be a good vertebrate candidate for sediment testing.

Although both laboratory and field bioassays can provide a measure of toxicity, it appears that laboratory assays enhance bioavailability, for instance, through disturbance caused by sediment handling and animal-driven resuspension. The results from laboratory assays may be useful for more baseline mechanistic studies focusing on the effects of sediment-bound contamination and also for those that seek a more conservative approach to establish toxicity thresholds. Nevertheless, field studies provide more ecologically relevant data, which is likely more adequate for standard regulatory purposes. Still, both types of assays have assets as well as handicaps. Noise variables are a bigger problem in the field, deriving mostly from the cyclically shifting environment (a major feature in an estuary). In the future, it would be important to apply the same methodologies to further sites in the estuary and test improved laboratory procedures, e.g. by using model sediments spiked with mixtures of contaminants or testing with sediment elutriates. Such assays are likely to reduce experimental noise and, simultaneously, constitute an approach to more standardized testing procedures. It would also be important to enhance knowledge on the biology of complex contaminant interactions, especially those prone to cause chronic disease, as for low to moderate environmental contamination, namely by performing cell-based assays using vertebrate cell cultures from fish, mice or even humans, combined with state-of-the art molecular and cytological tools.

The present work provided many important novel insights on classical biomarkers, improvement of techniques and new potential biomarkers and mechanisms of toxicity of isolated or combined

xenobiotics. The information produced is therefore relevant for all those who strive to make sense out of naturally-occurring combinations of contaminants as in estuarine sediments but also shows that research is still needed to fully disclose the effects of low to moderate levels of background pollution to aquatic biota and fish in particular.

# Annexes

## Annex 1. Peer-reviewed publication list

The following articles have been produced on the course of this PhD thesis:

| Section | Reference |
|---|---|
| 2.1. | **COSTA, P.M.** & COSTA, M.H. (2007). Genotoxicity assessment in fish peripheral blood: a method for a more efficient analysis of micronuclei. *Journal of Fish Biology*, **71**(SA):148-151. |
| 2.2. | **COSTA, P.M.** & COSTA, M.H. (2008). Biochemical and histopathological endpoints of *in vivo* cadmium toxicity in *Sparus aurata*. *Ciencias Marinas*, **34**(3):349-361. |
| 3.1. | **COSTA, P.M.**, LOBO, J., CAEIRO, S., MARTINS, M., FERREIRA; A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (2008). Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. *Mutation Research*, **654**(1):29-37. |
| 3.2. | **COSTA, P.M.**, CAEIRO, S., DINIZ, M.S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (2009). Biochemical endpoints on juvenile *Solea senegalensis* exposed to estuarine sediments: the effect of contaminant mixtures on metallothionein and CYP1A induction. *Ecotoxicology*, **18**(8):988-1000. |
| 3.3. | **COSTA, P.M.**, DINIZ, M.S., CAEIRO, S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (2009). Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: a weighted indices approach. *Aquatic Toxicology*, **92**(3):202-212. |
| 3.4. | **COSTA, P.M.**, CAEIRO, S., DINIZ, M.S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (2010). A description of chloride cell and kidney tubule alterations in the flatfish *Solea senegalensis* exposed to moderately contaminated estuarine sediments. *Journal of Sea Research*, **64**(4):465-472. |

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| Section | Reference |
|---|---|
| 4.1. | **COSTA, P.M.**, NEUPARTH, T., CAEIRO, S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À., & COSTA, M.H. (2011). Assessment of the genotoxic potential of contaminated estuarine sediments in fish peripheral blood: laboratory versus *in situ* studies. *Environmental Research* **111**(1):25--36. |
| 4.2. | **COSTA, P.M.**, CAEIRO, S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (2011). Estuarine ecological risk based on hepatic histopathological indices from laboratory and *in situ* tested fish. *Marine Pollution Bulletin* **62**(1):55-65. |
| 4.3. | **COSTA, P.M.**, MIGUEL, C., CAEIRO, S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., DELVALLS, T.À. & COSTA, M.H. (*submitted*). Evaluation of stress-related gene transcription profiles in flatfish exposed to contaminated estuarine sediments. |
| 4.4. | **COSTA, P.M.**, CHICANO-GÁLVEZ, E., CAEIRO, S., LOBO, J., MARTINS, M., FERREIRA, A.M., CAETANO, M., VALE, C., J. ALHAMA, LOPEZ-BAREA, J., DELVALLS, T.À. & COSTA, M.H. (*submitted*). Hepatic proteome changes in *Solea senegalensis* exposed to contaminated estuarine sediments: a laboratory and *in situ* survey. |
| 5.1. | **COSTA, P.M.**, CHICANO-GÁLVEZ, E., LÓPEZ BAREA, J., DELVALLS, T.À. & COSTA, M.H. (2010). Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]pyrene. *Environmental Pollution*, **158**(10):3338-3346. |
| 6.1. | **COSTA, P.M.**, CAEIRO, S., VALE, C., DELVALLS, T.À. & COSTA, M.H. (*submitted*). Can the integration of multiple biomarkers with sediment geochemistry solve the complexity of sediment risk assessment? A case study with a benthic fish. |

## Annex 2. GenBank sequences

The following gene sequences have been discovered and deposited in the public-access GenBank database (www.ncbi.nlm.nih.gov/genbank) in the course of the research described in this thesis:

DEFINITION: *Solea senegalensis* metallothionein 1 mRNA, complete cds.
ACCESSION: GU946410

```
        1 tttgtgtctg ggctacctga tactcaagtt tttcaacaat gatattcctg aagggcttcg
       61 tcggctgcca ggacccaagc ccctgcccat tattgggaat gtgctggagg tgggcagcaa
      121 accttacctg agtctcactg aaatgggcaa gcgctacggc cacgttttcc aaatccagat
      181 tggcatgcgt cctgtggttg tgctgagtgg cagtgaaact gttcgacagg ctctcatcaa
      241 gcagggggat gatttttcgg gcaggcctga cctgtacagc tttcgcttca tcagcaatgg
      301 taagagtctg gccttcagca cagaccaggc cggtgtctgg cgtgcccgca gaaagctggc
      361 ctacagtgcc ctccgctcct ttgccaccct ggagggcacg accccacaat actcctgcgt
      421 tctagaggaa cacgtgtgca aagaaggaga gtatctgatc aacgagctca acactgtcat
      481 gaaggctgag ggatccttcg acccattccg ctacatagtt gtctctgtag ctaatgtgat
      541 ctgtggaatg tgctttggcc gacgctacga ccacgacgat caggagctgg a
```

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DEFINITION *Solea senegalensis* catalase mRNA, partial cds.
ACCESSION: GU946411

```
        1 ttatgacctt tgagcaggct gaaaagttcc agttcaaccc cttcgatctt accaaggttt
       61 ggtcccataa agaataccct ttgatccccg tgggcaaact tgttctcaac aggaacccag
      121 tgaactattt tgcagaggtg gagcagctgg cctttgaccc aagtaacatg ccaccaggca
      181 ttgagccaag ccccgacaaa atgcttcagg gtcgactctt ctcctacccg gacacacatc
      241 gacacagact gggtgccaac taccttcaga tcccagtcaa ctgcccattc agaacccgcg
      301 tggccaacta ccagcgcgat ggcccgatgt gtatgtttga caaccaaggt ggtgctccaa
      361 actactancc caacagcttc agtga
```

---

DEFINITION: *Solea senegalensis* CYP1A mRNA, partial cds.
ACCESSION: GU946412

```
        1 tttgtgtctg ggctacctga tactcaagtt tttcaacaat gatattcctg aagggcttcg
       61 tcggctgcca ggacccaagc ccctgcccat tattgggaat gtgctggagg tgggcagcaa
      121 accttacctg agtctcactg aaatgggcaa gcgctacggc cacgttttcc aaatccagat
      181 tggcatgcgt cctgtggttg tgctgagtgg cagtgaaact gttcgacagg ctctcatcaa
      241 gcagggggat gatttttcgg gcaggcctga cctgtacagc tttcgcttca tcagcaatgg
      301 taagagtctg gccttcagca cagaccaggc cggtgtctgg cgtgcccgca gaaagctggc
      361 ctacagtgcc ctccgctcct ttgccaccct ggagggcacg accccacaat actcctgcgt
      421 tctagaggaa cacgtgtgca aagaaggaga gtatctgatc aacgagctca acactgtcat
      481 gaaggctgag ggatccttcg acccattccg ctacatagtt gtctctgtag ctaatgtgat
      541 ctgtggaatg tgctttggcc gacgctacga ccacgacgat caggagctgg a
```

---

DEFINITION: *Solea senegalensis* glutathione peroxidase (GPx) mRNA, complete cds.
ACCESSION: HM068301

```
        1 cagatgaacg agctgcactg tcgttattcc tcccaaggac ttgttattct gggtgtcccc
       61 tgcaatcagt ttggacatca ggagaacgcc aaaaacgatg aaatcctaag atctctgaag
      121 tatgtccgtc cagggaatgg ctttgaacca aagtttcagc ttcttgaaaa ggtggatgtg
      181 aacggagcag atgcacaccc cttgtttgtc tatctgagag aaaagctccc atttcccagt
      241 gataatacca tggctctcat gaccgatcca aagttcatca tttggagtcc agtgtgcagg
      301 aatgacgttg cttggaactt cgagaagttc ctggtcagcc ctgatggaga accctacaag
      361 cgctacagca gaaatttcct gaccatgaat cttgaggcag atattaaaga gctacttcag
      421 agggcgaagt aa
```

---

DEFINITION: *Solea senegalensis* cysteine-aspartic acid peptidase 3 (caspase 3) mRNA, partial cds.
ACCESSION: HQ115741

```
        1 gcatcatcat caacaacaag aactttgacg aaaggacagg gatgaacgta cgcaacggca
       61 cggaccgtga cgcgggcgag ctgttcaaat gcttcaagac cctgggcttc gatgtcttca
      121 tctacaacga tcagacctgt gagaagatgg aatgtcttct cagagaagcc tcggaggaag
      181 accatagtga cagctcgtgt ttcgcctgta tcctgctaag ccacggcgag gagggcatga
      241 tctacggcac agatggagcc atgcccatca agaccatgac ctcactgttc agggggggaca
      301 tgtgcaaaag cttagttgga aagcctaagc tgttctttat ccaggcttgt ag
```

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